THE FURTHER CHARACTERIZATION OF A GOITROGEN IN PEARL MILLET (*Pennisetum americanum* (L.) Leeke)

by

DIANNE MARIE BIRZER

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Approved by:

Carol F. Kupferstein
Major Professor

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INTRODUCTION

Pearl millet (Pennisetum americanum (L.) Leeke) is one of the most important food crops in the semi-arid tropics (1). Of all the cereals, it is one of the most drought resistant, and it grows well in sandy soils. It is often grown as a crop of last resort, and failure of the crop may mean starvation.

Nutritionally, pearl millet is good food. The protein efficiency ratio (PER) for pearl millet is generally higher than the PER's for wheat or sorghum (2).

However, Osman and Fatah (3) in a study of endemic goiter in Sudan, implicated pearl millet as a possible contributory factor. Further studies (4, 5) indicated that rats fed millet developed goiter-like histological changes in their thyroids and abnormal thyroid hormone patterns.

Klopfenstein et al. (6, 7, 8) showed that feeding rats pearl millet induced typical goiter-like changes in thyroid histology, including enlargement of the thyroid colloid follicles, hyperplasia, and flattening of the epithelial cells, whereas thyroids of sorghum-fed rats

were normal. They also showed that thyroxine (T_4) concentrations were higher and that triiodothyronine (T_3) concentrations were lower in millet-fed rats than sorghum-fed rats.

That work also indicated that the goitrogenic activity was associated with both the millet bran and endosperm fractions of the grain, that it was not associated with the grain's high iron content, and that it could not be prevented by dietary iodine supplements. Fermenting the millet did not seem to enhance or destroy the goitrogenic activity, while autoclaving the millet appeared to alleviate the effect. Histological data indicated that the goitrogen was at least somewhat water soluble.

The purpose of this study was to further characterize the pearl millet goitrogen.

LITERATURE REVIEW

The Millets

History

The millets, along with sorghum grain (Sorghum bicolor (L.) Moench), are the most important food crops in the semi-arid tropics (1). Pearl millet (Pennisetum americanum (L.) Leeke), also known as bulrush millet, or bajra in India, is the most widely grown of all the millets. Both millet and sorghum originated in Africa and today are grown throughout Africa and in the drier parts of India as food crops. In the United States and other developed countries, little acreage is devoted to millet grains, and sorghum is grown almost entirely as a feed crop; but in developing countries most of the millet and sorghum is consumed as food. Both of these cereals are tolerant to dry conditions. Millet can survive with less total rainfall than sorghum, but sorghum can survive better in intermittent drought, since it can become temporarily dormant. The millets grow well in dry sandy soil and in many areas are grown as a crop of last resort. Failure of the millet or

sorghum crop may mean starvation in many areas of the world.

Nutrition

As is true of most cereals, pearl millet and sorghum are good foods, but their proteins contain low concentrations of some essential amino acids. The limiting amino acids are lysine and the sulfur-containing amino acids (2). Tryptophan content also tends to be low. Sorghum's leucine content is generally high, a circumstance which may be responsible for the high incidence of pellagra in people who subsist mainly on sorghum (2). Hulse et al. (1) reported that the protein content of pearl millet can range between 7.8 and 19.3%. Sorghum has a slightly lower protein content, ranging between 7.1 and 14.2%.

In general, the protein efficiency ratio (PER) is higher for pearl millet than for wheat or sorghum (2). Klopfenstein et al. (9) showed that pearl millet diets, supplemented with vitamins and minerals produced higher weight gains than isonitrogenous sorghum diets similarly supplemented. The presence of tannins in some sorghums further reduces their nutritive value (1). Tannins are known to precipitate dietary proteins, making the

proteins indigestible, and to inhibit digestive enzymes as well (10).

Pigments

The color of pearl millet varies from off-white to dark brown, but the most common, and generally preferred, color is slate gray (2). Reichert (11) identified some of the grey pigments in pearl millet as the C-glucosylflavones vitexin, glucosylvitexin, and glucosylorientin. Reichert and Youngs (12) used acid to bleach these pH-sensitive, polyphenolic pigments from the millet. This allowed for the production of a creamy white millet flour which is more acceptable to consumers than the gray colored product obtained without bleach.

Sorghum is known to contain a number of polyphenolic, or flavonoid, compounds which produce the characteristic reds, yellows and browns of the grain. The condensed tannins, which are polymers of flavonoid compounds, are probably the best known. Other flavonoids, especially anthocyanins, also exist in sorghum. Nip and Burns (13) identified the anthocyanins apigeninidin and luteolinidin as the red color producing pigments in red sorghum. In white sorghum the yellow-colored glycosides of these compounds have been

identified (14). Food products made from brown sorghums are generally considered unacceptable by consumers (15).

Phenolic Acids

Reichert(11) mentioned that three phenolic acids are present in alkali extracts of pearl millet, one of which he identified as ferulic acid. Reddy (16) identified three phenolic acids in the methanol extract of pearl millet: p-hydroxybenzoic acid, ferulic acid and cinnamic acid.

Hahn et al. (17) have identified a number of phenolic acids in sorghum including cinnamic, ferulic, caffeic, vanillic, gallic, protocatechuic, p-hydroxybenzoic and p-coumaric acids.

Food Uses

Pearl millet and sorghum have very similar food uses. They are frequently made into flat breads, such as roti or chapatti in India, or kisra, a fermented Sudanese flat bread (2). In some areas cous cous is made of millet. The grain may also be fermented and consumed as a thick, nutritious beer (2).

Both pearl millet and sorghum are frequently consumed as porridges, which may be prepared in a variety of ways. Some are fermented and others, such as

tô, may be acidified with tamarind or lemon (18). In some areas alkaline tô is produced by the addition of wood or peanut hull ash extract.

Goiter

The disease condition known as goiter occurs when the thyroid gland tries to adapt to the inadequate production or utilization of the thyroid hormones (19). The thyroid responds to this lack by histological changes, primarily enlargement of the thyroid colloid follicles, and proliferation of the surrounding epithelial cells.

The Thyroid Gland and Thyroid Hormones

The thyroid gland is a small, flat, bi-lobed structure (20). The lobes, usually of fairly equal size and symmetrical, lie on either side of the trachea and are connected by a small, thin isthmus. The function of the thyroid is to accumulate iodine and to produce and store the thyroid hormones.

In adult vertebrates, the thyroid is made up of many small spherical vesicles, called follicles, which usually consist of a single layer of epithelial cells arranged around a mass of colloid (20). The colloid is,

in part, made up of the protein thyroglobin which represents the storage form of the thyroid hormones. Thyroxine (T_4) is produced from two tyrosine residues which are present in thyroglobin. The tyrosine is first iodinated to form monoiodotyrosine (MIT), then to diiodotyrosine (DIT). Two DIT molecules are then enzymatically combined, with the elimination of one tryrosine side chain, to form thyroxine (T_4). T_4 is the more abundant thyroid hormone, but triiodothyronine (T_3) is the more active form. T_3 is produced mainly by the deiodination of T_4 in such peripheral tissues as the liver and the kidneys. It can also be produced by the condensation of MIT and DIT.

A number of physiological functions are regulated by the thyroid hormones (20). These include metabolic rate, growth and development, and metabolism of fats, carbohydrates, proteins and other nutrients.

Endemic Goiter

Endemic goiter can be defined as the common occurence of enlarged thyroid glands in the population of a particular locale (20). Goiter occurs in response to insufficient production of thyroid hormone by the epithelial cells lining the colloid follicles. The

cells increase in size and number with the result being larger follicles. The epithelial cells may begin to infold and eventually may replace the colloid inside the follicles. This condition is known as hyperplasia. Over time, there is an increase in the size of the thyroid.

Very large, goitrous thyroids have been reported to cause suffocation (19). The incidence of thyroid cancer in areas were goiter is endemic is about five to six times greater than it is in other areas. Also, in areas where goiter is endemic, cretinism is common (21). Cretinism is characterized by mental and physical retardation and is especially common in children whose mothers had goiter during pregnancy.

Causes of Goiter

The most common cause of goiter is inadequate dietary iodine (19). In recent years intensive efforts by world health organizations to distribute iodized salt and oil to areas of endemic goiter have helped reduce the incidence of iodine-deficiency goiter.

As goiter due to iodine deficiency decreased, it was noticed that, in some areas, endemic goiter persisted despite adequate dietary iodine (19). This

led to the discovery of goitrogens, which are dietary or environmental chemicals that can cause goiter by interfering with thyroid function. Goitrogens may be chemicals in the food or water, either natural constituents or contaminants. Some goitrogens are produced during the digestion of food components. Some medications can also be goitrogenic.

A number of different chemical compounds have been identified as goitrogens (22). One general group of goitrogens is the thiocarbamides, which contain a sulfur, carbon and nitrogen grouping (22). Thiourea, thiouracils, aminothiazoles and mercaptoimidazoles are included in this group. They act by preventing the incorporation of iodide into organic compounds. The exact mechanism of inhibition is not fully understood, but these sulfur-containing compounds might act by inhibiting the enzymes involved in iodination of tyrosine or those that catalyze the condensation of the iodotyrosines. Although thiocyanate also contains a sulfur group, similar to the ones found in thiocarbamides, thiocyanate acts as a goitrogen by preventing the concentration of iodine by the thyroid. It also may inhibit iodide peroxidase activity. Iodide peroxidase catalyzes the oxidation of iodine to an active form which can then be incorporated into the amino acid tyrosine (20). The reaction catalyzed by

iodide peroxidase is proposed to be $H_2O_2 + 2\ I^- + 2\ H^+$ ----> 2 'Active I' + 2 H_2O (22). The 'active I' is then used to iodinate tyrosine. Some aromatic compounds, especially those containing electron-donating groups, are the other main type of goitrogen (23). These include p-aminobenzoic acid, resorcinol (1, 3-dihydroxybenzene) and phloroglucinol (1, 3, 5-trihyroxybenzene). Benzene rings that contain meta substituted electron-donating groups seem to be particularly active goitrogens. The theory behind the activity of these compounds is that they competitively bind the iodine in the body, making it unavailable for hormone synthesis. In such cases, additional dietary iodine usually overcomes the goitrogenic effect. It has also been suggested that these compounds inhibit peroxidase activity (22).

Dietary Goitrogens

A number of important dietary goitrogens have been identified in the past few years. One of the earliest foods to be identified as goitrogenic was cabbage. Other members of the Cruciferae family, including kale, mustard seed, and rapeseed, are known to have the same effect (24). The compound responsible for the goitrogenic action of these plants was identified as 5-

vinyl oxazolidine-2-thione (VTO) or goitrin. Goitrin and other isothiocyanates are released when myrosinase, an endogenous enzyme, acts on glucosinolates present in the plants (25). This occurs when the plant is ground or crushed during eating or processing.

Since the discovery of the goitrogen in rapeseed, low glucosinolate rapeseed varieties have been developed, enabling this oil seed to become an important cash crop in some areas. It is used to produce high protein meal for feed as well as oil (26).

Casava, an important staple in some parts of the world, is also known to contain a goitrogen; in this case, the cyanogenic glycoside linamarin (27). When linamarin is treated with acid or endogenous hydrolytic enzymes, which may be released during eating or processing, hydrogen cyanide (HCN) is produced. HCN is toxic and is metabolized by a reaction with thiosulfate, forming thiocyanate, which is a goitrogen. Adding excess iodine to the diet overcomes the goitrogenic effect of the thiocyanate. Also, proper processing of the cassava can remove most of the linamarin before it is ingested.

A number of other foods also contain cyanogenic glycosides (27). Linamarin, the goitrogen in casava, is also found in lima beans and other legumes. Amygdalin is found in almonds, apples, cherries and peaches.

While dhurrin is found in sorghum, especially in the young leaves, it is not present in the grain and is, therefore, not a threat to humans.

Peanuts are known to have a goitrogenic effect, even when large amounts of iodine are present in the diet (28). The goitrogen was identified as the anthocyanin, arachidoside (29, 30). This is a polyphenolic pigment which gives a red color to the peanut skin. Similar substances from cashews and almonds were found to have the same effect.

It was postulated by Mougdal et al. (30) that these compounds and the phenolic compounds formed during their digestion, such as phloroglucinol, act by forming stable iododerivatives containing large amount of iodine. In this way they would compete with tyrosine for available iodine. They also suggested that these compounds act by blocking the enzymatic binding of iodine to tyrosine, preventing the formation of MIT. In addition, these compounds seem to inhibit the uptake of inorganic iodine by the thyroid gland. It was shown that 50% less iodine was present in the thyroids of rats fed arachidoside than in the thyroids of control rats.

Konijn et al. (31) suggested that a goitrogen found in soybeans may be an oligopeptide or glycopeptide. Work by Suwa et al. (32) showed that treating soybean

curd with proteolytic enzymes did not remove the goitrogenic effect. Further work by Suwa and Kimura (33) suggested that at least one of the goitrogenic elements was soya-sapogenol-glycoside, a polyphenol. Recently, Tani et al. (34) reported that soybeans contain the polyphenolic glycosides genistin, daizein and daidzin.

The Goitrogenicity of Pearl Millet

Osman and Fatah reported that pearl millet may contribute to the endemic goiter found in some areas of Sudan (3). They examined 1604 school children for goiter and, at the same time, conducted a dietary survey. They concluded that a number of factors contributed to the high incidence of goiter in these areas, including low iodine intake, high sodium, potassium, and iron content in the water, low vitamin A in the diet and generally poor nutritional status. It was also noted that in villages, where pearl millet comprised a larger percentage of the diet, the incidence of goiter was higher than in the urban areas, where other grains are also consumed.

To further study this effect, Osman (4) fed young male Wistar rats pearl millet or sorghum diets. Rats fed

millet showed abnormal thyroid cells, hyperplasia of the thyroid follicles, and abnormal thyroid hormone patterns, while the thyroids and thyroid hormone patterns of sorghum-fed rats were unaffected.

Osman et al. (5) separated millet extract by paper chromatography and found that one spot responded positively to Grote's reagent, which is used as a general test for sulfur-containing compounds (35). Osman et al. (5) concluded that the compound was a thioamide, some of which are known to be goitrogens. They also reported high serum isothiocyanate concentrations in Sudanese girls with goiter. They suggested that the high blood concentrations of isothiocyanate were due to the ingestion of the thioamide present in the millet.

Klopfenstein et al. (6) also reported that pearl millet diets caused histological changes in rat thyroids, including enlarged follicles, hyperplasia and flattening of the epithelial cells. Thyroxine (T_4) concentrations were found to be higher and triiodothyronine (T_3) concentrations lower in millet-fed rats than in sorghum-fed rats.

From this and other work by Klopfenstein et al. (7, 8) some characteristics of the goitrogen were discovered. It was found that the goitrogen is

associated with both the pearl millet bran and endosperm fractions. Though some millets have a high iron content, which may promote goiter, low - iron millet also causes goiter-like responses in rats.

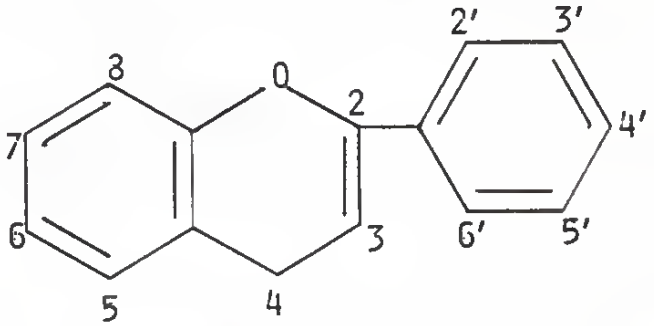
Iodine supplementation of millet did not prevent the goiter symptoms (9). This suggests that the goitrogen is not a thiocyanate or isothiocyanate because the goitrogenic effects of these chemicals can be prevented with iodine supplements.

It was found that fermenting the millet neither enhanced nor destroyed its goitrogenic effect. Autoclaved millet did not produce histological or hormonal changes to the same extent as untreated millet, suggesting the presence of a volatile or heat-labile compound. Histological data suggested that the goitrogen could be extracted from the grain with water.

Recent reports by Gaitan et al. (36) indicate that the polyphenolic pigments in the millet may be the goitrogenic agent. These are digested by gut bacteria producing resorcinol, dihydroxybenzoic acids and ferulic acid (37). Aromatic compounds such as these have been shown to have a goitrogenic effect (23). It has also been reported that millet contains a thiocyanate, which may contribute to the antithyroid activity (36).

Flavonoids

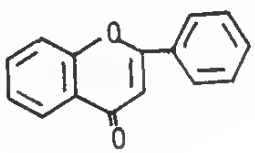
Flavonoids are polyphenolic compounds, very common in plants as red, blue, and yellow pigments (38). In combination with other compounds or minerals they can produce many other colors. The three most common types of flavonoids are the anthocyanins, the flavones, and the flavanols. They all have the same basic, three ring structure:



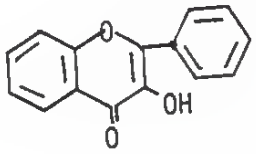
Anthocyanins are responsible for almost all reddish and blue colors in petals and leaves of plants (38). The variations in color are due to the number and/or position of hydroxyl groups on the rings, and their degree of methylation or glycosylation. The 3, 5, 3', and 5' positions are those most commonly hydroxylated or methylated. Glycosylation generally occurs at the 3, 5 and 7 positions. The most common sugars involved are glucose, rhamnose, galactose and arabinose, though others are also found. Since the aglycones, or anthocyanidins, are unstable, they are rarely found.

Anthocyanins are widespread in foods (38). A number have been reported in grain, including cyanidin-3-arabinoside and cyanidin-3-glucoside in barley. Cyanidin-3-arabinoside, apigeninidin-5-glucoside and luteolinidin-5-glucoside have been reported in sorghum leaves. In the sorghum grain, luteolinidin, apigeninidin-5-glucoside and kaempferol-3-rutinoside-7-glucuronide have been identified (13, 14).

The flavones and flavonols are similar in structure to the anthocyanins, but are more highly oxidized (38). They are responsible for yellow colors, and they also have a bluing effect when present with anthocyanins. They are often found as O-glycoside derivatives, as well as C-glycosides.



FLAVONE



FLAVONOL

The most common flavones are apigenin (4', 5, 7-hydroxyflavone) and luteolin (3', 4', 5, 7-hydroxyflavone) (38). The most common glycoflavones are vitexin (8-C-glucosylapigenin) and isovitexin (6-C-

glucosylapigenin). The glucoside analogs of luteolin are orientin and isoorientin. The flavone-C-glycosyl derivatives are widely distributed in the grasses, especially in the leaves. Flavonols are more common in other plant parts, they are rare in the leaves.

Reichert (11) has reported that vitexin, glucosylorientin and glucosylvitexin are present in pearl millet grain. Their presence produces an undesirable grey color in the flour when pearl millet is milled on a large scale for consumers (12). King has reported the presence of C-glucosylflavones in wheat germ (39). He was not able to positively identify the specific flavonoids present.

Proanthocyanidins are another important group of flavonoids (40). These are polymers of flavan-3,4-diols and flavan-3-ols, and are commonly called tannins. They are found in many plants, including the grain of some sorghum varieties. The high-tannin sorghums are important in some areas of the world because they are resistantto molds and to predation by birds (41). At the same time, since the tannins precipitate protein, these sorghums have a lower nutritional value than low-tannin varieties. Millet does not contain condensed tannins (42).

Functions of Flavonoids

Over the years, a number of functions have been suggested for the various flavonoid compounds. Some of these involve the plants that synthesize the flavonoids, others involve animals and other organisms which may come in contact with the flavonoids.

The most obvious function of the flavonoids is as pigments (38). In fruits and flowers these pigments attract insects, birds and other animals which pollinate the plants or help disperse seeds.

Flavonoids have been implicated in the regulation of plant growth (43). They may act as cofactors or interact with growth hormones to inhibit or promote their activity.

Recently, the flavone luteolin has been identified as an inducer for the nodulation genes of *Rhizobium meliloti* (44). These genes are responsible for stimulating host responses which allow nodule development in legume roots when they are invaded by nitrogen-fixing bacteria such as *R. meliloti*. This allows for symbiosis between certain legumes and some nitrogen-fixing bacteria.

It has been suggested that many flavonoids act in protecting the plant against attack by predators and

microorganisms (43). It is assumed that the high tannin content of some sorghums causes an astringent taste which repels birds (45). Hammerschmidt and Nicholson (46) showed that flavones produced in fungi-infected maize have a toxic effect on the pathogen. A variety of flavonoids have been recognized as antifungal and antibacterial agents produced by plants when attacked by these organisms (45).

Some investigators have suggested that the cytotoxic effects of the flavonoids are retained when ingested (45, 47). In this way, they may protect organisms eating foods rich in certain flavonoids from infection by microorganisms. This seems to be a property of highly methylated flavonoids.

Flavonoids have also been implicated as anticarcinogens (45, 47). This effect is attributed to their ability to induce an enzyme or enzyme system which detoxifies some carcinogens.

Flavones are antioxidants (47). Their presence in certain foods may protect other food components, such as unsaturated fatty acids or vitamins, from deteriorating on contact with air. Thus, they may act in some mixed dishes to prolong shelf-life, improve keeping quality and preserve taste.

Some flavonoids, especially the flavonols, have a

metal chelating effect (47). Their ability to chelate copper is especially important in biological systems. Because of this ability, some flavonoids may inactivate or inhibit copper-containing or copper-activated enzymes.

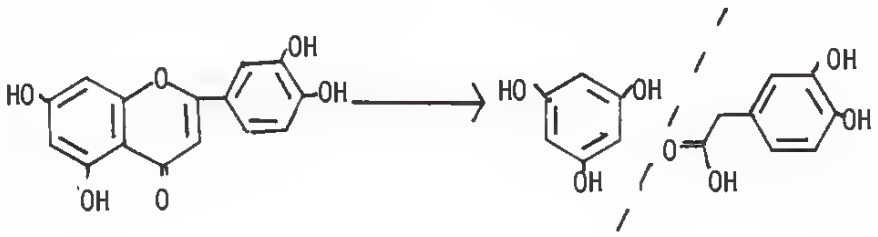
It has been shown that some flavonoids, especially catechins, anthocyanins and flavonols, can act as vitamin C synergists (47). Flavones have little or no effectiveness in this respect. The effect has been attributed to the fact that flavonoids are antioxidants and to their ability to chelate copper, which promotes the oxidation of vitamin C. Also, it has been theorized that vitamin C may bind with some flavonoids producing a stable storage form protected from oxidation.

Metabolism of Flavonoids

Most flavonoids are catabolized by microflora in the lower intestinal tract (47). The products vary depending on the type of flavonoid originally encountered.

Booth et al. (48) recognized that the flavone quercetin was degraded in humans, rats, rabbits and guinea pigs to 3,4-dihydroxyphenylacetic acid (DHPAA). DHPAA may then undergo methylation or dehydroxylation, forming other phenylacetic acids. Further work by Booth

et al. (49, 50) showed that many flavonoids are degraded by ring fission to phenylpropionic acids and hydroxyphenols. They also found that the original flavonoid was excreted in the urine of humans seven to fourteen hours after ingestion, indicating that some flavonoids are absorbed by the body without being metabolized.



RING FISION OF A FLAVONE TO
PHLOROGLUCINOL AND DHPAA

Griffiths and Smith (51) showed that some flavonoids are largely resistant to ring fission. Flavonoids are more susceptible to ring fission if they possess free 5- and 7- hydroxyl groups and a free 4'-hydroxyl group. They also showed that some of the unmetabolized flavonoids are absorbed by rats and humans.

Griffiths and Smith (52) showed that, in rats, ingestion of myricetin-3-rhamnoside resulted in the excretion of the aglycone, myricetin. Incubation of the rhamnoside with rat intestinal microflora also resulted

in the formation of the aglycone. It was also shown that in animals lacking intestinal microflora, little or no ring fission occurred. This indicated that most digestion of flavonoids was a result of microbiological activity; mammals do not seem to have the enzymes necessary for this process. Ingestion of apiin, apigenin-7-apiosylglycoside, led to the excretion of apigenin in the urine (51). In the same experiment the administration of other glycoflavones led to the excretion of the aglycone in the urine. This would indicate that some glycosides are converted to their aglyconesduring digestion.

After absorption the flavonoids are bound in the liver as glucuronides or sulfate conjugates which are then excreted into the urine or, more often, the bile (49). From the bile they pass back into the intestine where they may again be attacked by bacteria, liberating glucuronic and sulphuric acids and perhaps undergoing ring cleavage.

Pearl millet is an important staple in many parts of the world and, in general, it is a nutritious food. However, its goitrogenic properties are undesirable, since endemic goiter can cause mental and physical retardation. Unfortunately, pearl millet is most important in dry, under-developed areas of the world where few other food sources are available. Therefore,

it is important to identify the goitrogen present in pearl millet.

The purpose of this study will be to further characterize the pearl millet goitrogen. Since many goitrogens are known to be sulfur-containing compounds, a number of tests will be employed in an attempt to gain evidence for the presence of a similar sulfur-containing compound in pearl millet. Also, many phenolic compounds have been identified as goitrogens, and a number of phenolic compounds have been identified in pearl millet. To further characterize the goitrogen, pearl millet extracts containing possible goitrogens will be added to sorghum, which does not exhibit goitrogenic activity, and then used in a rat-feeding study.

A number of tests exist for sulfur-containing compounds, including the nitroprusside test, which is very general, and the ferric nitrate test, which is specific for thiocyanate and isothiocyanate ions. Recently, an enzyme assay has been developed which is very specific for compounds containing nucleophilic sulfur or nitrogen atoms. Many such compounds are also known to be goitrogens, making the assay useful in identifying possible goitrogenic compounds in pearl millet extracts.

MATERIALS AND METHODS

ASSAY FOR MONOXYGENASE ACTIVITY

A flavin (FAD)- containing, mixed - function monooxygenase, which catalyzes the oxidation of a large number of compounds that contain nucleophilic nitrogen and sulfur atoms, has been purified and characterized (53). The sulfur is oxidized in the presence of oxygen and NADPH is converted to NADP, a reaction that can be monitored spectrophotometrically. Methimizole is an especially good substrate for this enzyme, as are other thiocarbamides and thioamides. Since many such compounds are found in nature and some have been shown to be goitrogens, pearl millet extracts were assayed to determine whether they contained a nucleophilic sulfur- or nitrogen-containing compound which was a substrate for the flavin-containing monooxygenase. Phosphate buffer extracts of pearl millet were prepared, mixed with purified flavin-containing monooxygenase and other necessary components, and then spectrophotometrically assayed for the disappearance of NADPH. Disappearance of this compound would indicate the extract contained a substrate for the enzyme. Such a substrate would be a likely candidate for a goitrogen. Since animals fed

sorghum grain do not develop goitrous symptoms, extracts of that grain were used as a control.

EXTRACTION PROCEDURE Since lipids inhibit the monooxygenase, finely ground pearl millet and sorghum were defatted by a 12-hour Soxhlet hexane extraction. Twenty grams of the defatted grain were then shaken with 100 ml potassium phosphate buffer, then filtered through Whatman No. 42 fluted filter paper. Two phosphate buffers were used, an acid buffer, pH 5.6, (0.024 M) and a basic buffer, pH 8.4, (0.024 M). Three different buffer extraction procedures were followed: A, B, and C. In Procedure A, the grain was extracted with the pH 5.6 phosphate buffer only; in Procedure B, the grain was extracted with the pH 8.4 phosphate buffer only; and in Procedure C, the grain was sequentially extracted with the pH 5.6 phosphate buffer, followed by the pH 8.4 phosphate buffer.

Five milliliters of each extract were then mixed with 1.0 ml of 0.3 M perchloric acid, and the precipitated protein was removed by centrifugation. The clear supernatant was adjusted to pH 8.4 with 6 N, then 1 N, sodium hydroxide (NaOH). The protein-free extracts were then frozen until used.

The above extraction procedure was also followed using pearl millet that had first been treated by 12-

hour Soxhlet extraction using chloroform as a solvent instead of hexane. This was done in an effort to remove some of the more polar lipids from the final extracts, since it was thought that these compounds might inhibit the monooxygenase activity (54).

In an effort to increase enzyme activity, a more concentrated extract was prepared. Sixty grams of hexane defatted grain were extracted with 100 ml phosphate buffer as described in Procedure C. Five ml of the extract were treated with 0.3 M perchloric acid and then adjusted to pH 8.4 as described in Procedure 1.

ENZYME ASSAY PROCEDURE A 0.02 ml aliquot of the final 5 ml extracts from each of the procedures described above was mixed with 0.05 ml of Tricene/EDTA buffer, pH 8.4, (aerated and stored at 4°C), and 0.002 ml of NADPH (5 mM). Final concentrations were 0.1 M Tricene, 1.0 mM EDTA and 0.1 mM NADPH. N-octylamine (8 microliters, final concentration 2.4 mM) was added as an activator (53). The assay was performed at pH 8.4, for maximum enzyme activity. Two microliters of EDTA buffer solution containing partially purified flavin (FAD)-containing mixed-function monooxygenase was added to the buffer-extract solution. The partially purified enzyme was supplied by Dr. Thomas Roche, Department of

Biochemistry, Kansas State University. He had recieved it as a gift from Dr. D. M. Zeigler (53). The final volume was made to 1.0 ml with deionized water. Assays were performed at 37°C. The disappearance of NADPH was followed with a Beckman DU-8 computing spectrophotometer at 340 nm. Readings were taken every minute for 15 minutes.

CHEMICALS All chemicals used in this assay were were obtained from Sigma Chemical Company (St. Louis, MO) except the chloroform, reagent grade, which was purchased from Fisher Scientific (St. Louis, MO).

NITROPRUSSIDE TEST FOR SULFUR-CONTAINING COMPOUNDS

Many known goitrogens are sulfur -containing compounds (22). In an attempt to find evidence for the presence of such a compound in pearl millet extracts Grote's procedure (35) was used. This is a very general, qualitative assay which tests for large quantities of any compounds containing a C-SH, C=S, or C-S-S-C bond.

TEST PROCEDURE The test solution was prepared as follows: Two grams of sodium nitroferricyanide ($Na_2[Fe(CN)_5NO]$) was dissolved in 40.0 ml distilled water at room temperature and 2.0 g hydroxylamine hydrochloride was added, followed by 4.0 g sodium

bicarbonate. After evolution of gas ceased, 8 drops of bromine were added. Excess bromine was removed by aeration and the solution was filtered through a Whatman No. 4 filter paper. The filtrate was diluted to 100 ml with distilled water.

To test, an excess amount of sodium bicarbonate (about 0.5 g) was added to 2-3 ml of buffer extract or standard solution, then about 0.5 ml of the test solution was added. An instant purple-red color was considered positive for compounds containing C-S-H while an intense blue or green color was considered positive for the presence of C=S containing compounds.

EXTRACTS Pearl millet and sorghum extracts prepared for the assay for monoxygenase activity, Extraction Procedure C, were used.

In an effort to determine if any sulfur-containing compounds were destroyed or lost during the extraction procedure, a separate extraction procedure was done for this test. Six grams each of full-fat or Soxhlet hexane defatted pearl millet were shaken with 25 ml extractant for 1 hour. The 3 extractants used were: distilled water, 0.024 M phosphate buffer, pH 8.4, or 0.1 M acetate buffer, pH 4.5. Sodium hydroxide (1.5 ml, 1N) was added to half the samples and all samples were shaken 5 minutes longer. Sodium hydroxide was added

because alkali treatment is known to release thiocyanate when attached to other compounds such as p-hydroxybenzoate (55). Samples were filtered through Whatman No. 42 filter paper. The same procedure was repeated with 2 ml of thiocyanate solution added to the grain-liquid mixture before shaking. The thiocyanate was added as an internal standard to determine if any active compounds were being destroyed by the extraction procedure.

STANDARDS Solutions of ammonium thiocyanate, thiourea, methimazole, and 5-propyl-2-thiouracil were prepared in water or the appropriate buffer.

FERRIC NITRATE TEST FOR ISOTHIOCYANATE AND THIOCYANATE ION

Some plants such as rapeseed and mustard seed are known to contain compounds that can form, on hydrolysis, thiocyanates or isothiocyanates which can act as goitrogens when ingested (25). The ferric nitrate procedure of Josefsson was used to test for the presence of isothiocyanate and thiocyanate ions (55).

TEST PROCEDURE Fifteen ml of extract or standard solution were treated with 15 ml of 10% trichloroacetic acid and any precipitate filtered off. To a 3 ml aliquot, 3 ml of 0.4 M ferric nitrate in 1N nitric acid was added. A dark red color indicated a positive

reaction for the presence of thiocyanate or isothiocyanate. Two drops of 5% mercuric chloride were added. This destroys the ferric thiocyanate and isothiocyanate, resulting in the disappearance of the red color.

EXTRACTS The extracts used in the nitroprusside procedure described above were also used in this procedure.

CYANIDE TEST

Some foods such as casava and lima beans are known to contain cyanogenic glycosides which can produce cyanide when processed or eaten (27). The cyanide is detoxified in the body by a reaction with thiosulfate, producing the goitrogen thiocyanate. In an effort to determine if a cyanide-containing compound was present in pearl millet, the following test was used.

TEST PROCEDURE Sodium hydroxide (0.5 ml, 0.1 N) was placed in the inner chamber of a Conway diffusion dish. Three milliliters of 10% sulfuric acid (H_2SO_4) were added to one side of the outer chamber. On the other side 1 ml water and 1 ml standard solution, or 1 g ground sorghum or pearl millet was placed. The dish was covered and swirled thoroughly to mix the acid with the

grain, and the sample was then allowed to sit for 1 hour at room temperature. Trichloroacetic acid (20%, 0.5 ml) was added to the center chamber, quickly followed by 1 drop bromine water. This was stirred with a glass rod and 0.2 ml arsenite solution was added. To a 1 ml aliquot of the mixture from the center chamber, 1.5 ml benzidine-pyridine reagent was added. A red-orange color was positive for cyanide.

REAGENTS Reagents were prepared as follows.

Arsenite Solution. One gram sodium meta-arsenite ($NaAsO_2$) was dissolved in water, then made to 100 ml. Concentrated H_2SO_4 (0.5 ml) was added to acidify the solution.

Pyridine-Benzidine Solution. Fifteen milliliters of pyridine hydrochloride solution was added to 3 ml of benzidine hydrochloride solution and mixed. The solution was stored in the refrigerator in a stoppered bottle. Fresh solution was made every few days, as needed.

Benzidine Hydrochloride Solution. Four grams of benzidine dihydrochloride were added to 100 ml of water in a brown glass-stoppered bottle. The solution was shaken until saturated, then allowed to stand. The supernatant was used. The solution was stored in the refrigerator.

Pyridine Hydrochloride Solution. Sixty milliliters of pyridine was added to 40 ml distilled water in a

glass-stoppered bottle. Ten milliliters of concentrated HCl was quickly added and the solution stirred. The solution was stored in the refrigerator.

Cyanide Standard. Twenty-five milligrams of potassium cyanide were dissolved and then made to 100 ml in 1 N NaOH. The solution was kept in the refrigerator. One milliliter of this solution was diluted to 100 ml for a working standard.

SPECTROPHOTOMETRIC SCANS

Spectrophotometric scans of buffer solutions of the known goitrogens methimazole, indole, thiocyanate and thiourea were compared with those of pearl millet, and sorghum. Buffer extracts of both grains were made and compared to each other in an effort to determine whether similar compounds were present in the pearl millet.

PROCEDURE Extracts and standards were scanned on a Varian DMS 80 UV-Visible double-beam spectrophotometer (Varian Associates, Palo Alto, CA) from 350 - 490 nm at 50 nm/ minute against the appropriate extraction buffer blanks.

STANDARDS Methimazole, indole, thiocyanate and thiourea were dissolved in 0.024 M phosphate buffer, pH 8.4 for use as standards.

SAMPLES The pearl millet and sorghum extracts from Extraction Procedure C for monooxygenase activity were scanned.

GRAIN USED IN PROCEDURES ABOVE

For the assay for monooxygenase activity and the tests for the presence of sulfur- containing compounds, a blend of pearl millet lines grown in Hays, Kansas and Garden City, Kansas in 1984 was used. Sorghum was a locally grown, low-tannin variety obtained from a commercial elevator in Manhattan, Kansas. A Varco coffee mill was used to finely grind the grains.

FEEDING STUDY

GRAIN The pearl millet used was a bulk sample obtained from the Agricultural Experiment Station in Hays, Kansas. The sorghum grain was a locally-grown, low-tannin variety. Both grains were ground in a Ross Experimental Mill with corrugated rolls set at 0.016 inch.

DIETS The diets for the rat-feeding study were designed to determine whether the compounds contained in a methanol extract of pearl millet were responsible for

its anti-thyroid activity and to assess their general nutritonal impart. The extract was rich in flavonoids, as well as other phenolic compounds. The effects of a high concentration of ferulic acid were also investigated.

Diet I (Sorghum Control). Sorghum grain + 2% vitamin diet fortification mix (Table 1) + 4% mineral mix (Table 2) + 3% soybean oil (purchased at a local supermarket). This diet was used as a control, since sorghum-fed animals have not been found to develop goiter-like thyroid histology or thyroid hormone abnormalities (6, 7, 8, 9).

Diet II (Millet Control). Millet grain + 2% vitamin mix + 3% mineral mix. This diet was used to observe the goiter-like thyroid histological and hormonal changes that have been reported to develop in millet-fed animals and to compare any changes that might result from this diet with any that that occur as a result of the other dietary treatments.

Diet III (Sorghum + 1X millet extract). Sorghum grain + pearl millet methanol extract + 2% vitamin mix + 4% mineral mix + 3% soybean oil.

Pearl millet has been found to contain the flavones glucosylvitexin, glucosylorientin and vitexin (11). Peanuts have been reported to contain a flavonoid that acts as a goitrogen (28, 29, 30). Polyphenolic

TABLE 1

VITAMIN MIXTURE COMPOSITION* (g/Kg)

Vit A Acetate (500,000 IU/g)	1.980
Vit D_2 (850,000 IU/g)	0.138
Vit E Acetate (500 IU/g)	11.000
Ascorbic Acid	49.500
Inositol	5.500
Choline Bitartrate	227.700
Menadione	2.475
p-Aminobenzoic Acid	5.500
Niacin	4.675
Riboflavin	1.100
Pyridoxine HCl	1.100
Thiamine HCl	1.100
D-Calcium Pantothenate	3.300
Biotin	0.099
Vitamin B_{12}	0.015

*ICN Vitamin Mixture No. 2, Nutritional Biochemicals, Cleveland, OH

TABLE 2

MINERAL MIXTURE COMPOSITION* (%)	
Sodium chloride	13.930
Potassium biphosphate	38.900
Magnesium sulfate (anhydrous)	5.730
Calcium carbonate	38.140
Ferrous sulfate	2.700
Manganese sulfate	0.401
Potassium iodide	0.078
Zinc sulfate	0.055
Cupric sulfate	0.048
Cobaltous chloride	0.002

*U.S.P. XVII Salt Mixture, Nutritional Biochemicals, Cleveland, OH

compounds in soybeans have also been implicated as goitrogens (32, 33) and reports have suggested that the flavones in pearl millet act as a goitrogen (36, 37). In an effort to determine if the pearl millet flavones are goitrogenic, they were extracted in methanol and the extract added to sorghum.

The methanol extract was prepared as follows. Ground pearl millet was defatted by hexanes in a Soxhlet extraction for 12 hours. The defatted grain was then mechanically stirred with methanol for one hour at room temperature, using a solvent-to-grain ratio of 2:1 (vol:wt). The methanol was removed from the grain by vacuum filtration using a Buchner funnel and Whatman No. 4 filter paper. Fresh methanol was added to the grain and the procedure repeated. The extracts were combined and reduced to approximately 1/5 their original volume in a rotary evaporator. The extracted pearl millet was retained for use in Diets VI and VII. The concentrated methanol extract was mixed with an amount of sorghum equivalent to the amount of pearl millet from which it had been extracted. The sorghum was allowed to air dry for 24 hours to remove residual methanol. The majority of the methanol evaporated within 2-3 hours and the grain was stirred a few times to facilitate the process.

Diet IV (Sorghum + 2X millet extract). Sorghum grain + pearl millet methanol extract, as in Diet III, +

2% vitamin mix + 4% mineral mix + 4% soybean oil. Pearl millet was extracted with methanol as described for Diet III, and the extract was mixed with an amount of sorghum equivalent to 1/2 the amount of pearl millet from which it had been extracted, then allowed to air dry.

This diet was prepared in an effort to determine if there was a dose-related goitrogenic response to components of the methanol extract.

Diet V (Sorghum + wetted millet extract). Sorghum + methanol extract from wetted pearl millet + 2% vitamin mix + 4% mineral mix + 4% oil. Enough tap water was added to the ground pearl millet to thoroughly wet it, approximately 1 g grain : 1 ml water . The grain was allowed to dry in an air current for 24 hours with frequent stirring, then spread out and dried in the sun for 6 hours. This grain was then extracted with methanol as described for Diet III and the extract mixed with an amount of sorghum equivalent to the amount of pearl millet from which it had been extracted, then allowed to air dry.

When pearl millet is moistened and then allowed to dry, it develops an unpleasant odor. The precursors of the odor-causing compounds were identified as the flavones glucosylvitexin, glucosylorientin, and vitexin (16). In preparing this diet, the millet was wetted

before extraction to determine if the odor-causing changes were related to the goiter-like thyroid histological and hormonal changes observed in millet-fed rats.

Diet VI (Extracted Pearl Millet). Methanol-extracted pearl millet + 2% vitamin mix + 4% mineral mix + 4.3% soybean oil. The methanol- extracted pearl millet retained after preparation of Diets III and IV was used.

This diet was prepared to determine if removing the flavones and other methanol-soluble compounds from the pearl millet would alleviate the goiter-like response that has been observed in millet-fed rats.

Diet VII (Extracted pearl millet + ferulic acid). Methanol extracted pearl millet + 2% vitamin mix + 4% mineral mix + 4.3% fat + 0.28% ferulic acid. Purified ferulic acid, obtained from Sigma Chemical Company, was added to the methanol-extracted pearl millet retained from preparing Diets III and IV. This diet was calculated to have approximately 280 mg ferulic acid/ 100 g grain.

Reichert (11) reported that pearl millet contained a high concentration of ferulic acid, about 158 mg/100 g grain, whereas its concentration in sorghum is much lower (17). Though ferulic acid has not been reported to cause goiter, a number of phenolic compounds have

been reported to do so (23). Very large polyphenolic compounds, such as tannins, are also known to act as anti-nutrients, presumably by precipitating proteins (41). Little has been written about the anti-nutrient effects of smaller phenolic compounds, but they are assumed to have no effect (41). It is known that they can have an anti-bacterial and anti-fungal effect. This diet was prepared to determine the effect of a high dietary concentration of ferulic acid on the thyroid histology and hormone pattern of rats, as well as any possible anti-nutrient effects.

ANALYSIS OF DIETS The diets were designed to be isocaloric, but not isonitrogenous. The soybean oil was added to the diets to make them all equal in fat content. The mean percents protein of the pearl millet and sorghum diets were found to be 15.06 ± 0.37% and 9.12 ± 0.37%, respectively. Fat and ash contents of all the diets were 6.4 ± 0.33% and 5.1 ± 0.24%, respectively.

PROCEDURE Seven groups, each containing 9-10 male Wistar rats (Charles River Breeding Laboratory, Wilmington, MA), weighing 85 ± 15 g, were individually caged and allowed free access to feed and water during the six-week feeding period. Weight gain was measured weekly, feed consumption records were kept and feed

efficiency ratios calculated. After six weeks, the animals were lightly ether-anesthetized and about 2 ml of blood were withdrawn from their hearts. After clot formation at room temperature, the serum was separated by centrifuging in the cold (5°C) at 3000 X g for 30 minutes. Serum was stored for several days at -20°C until being radioimmunoassayed for thyroxine (T_4) and triiodothyronine (T_3) (Animal Health Diagnostic Laboratory, East Lansing, MI).

On the day following blood sampling, again under ether anesthesia, tracheolaryngeal sections with adhering thyroid glands were removed and placed in 10% buffered, neutral formalin. Sections were embedded in paraffin, sectioned at 6 microns, stained with hemotoxylin and eosin (H & E) and evaluated microscopically. Some samples were also treated with von Kassa, alizarin red, or trichrom stains for further evaluation.

Duncan's Multiple Range Test (54) with analysis of variance procedure was used to evaluate the data.

PAPER CHROMATOGRAPHY

PROCEDURE Descending paper chromatography, as described by Mabry et al. (57), was used to separate the flavonoids in the methanol extracts of pearl millet. The R_f's obtained in the paper chromatography of flavonoids in various solvent systems are also important in the identification of these compounds. Whatman 3 MM chromatographic paper, 48 X 57 cm (Fisher Scientific) was cut into strips 19 X 57 cm. Pearl millet methanol extracts, prepared as described for Diets III and IV in the Feeding Study, were stored at -20^{o} for at least 24 hours to precipitate and remove some interfering sugars (11) and were then concentrated to about 1/2 their original volume under nitrogen. The extracts were then applied in a thin line, approximately 2 mm wide, 5 cm from the top, along the 19-cm edge of the paper. The paper was folded down along this same edge about 2 cm from the top and then back again so that it fit easily and squarely into the solvent trough containing 15% glacial acetic acid (HOAc). The paper was developed in a round glass chromatocab, 58 cm high and having a radius of 29.5 cm, until the solvent was about 5 cm from the bottom edge, (about 5-1/2 to 6 hours). UV light (366 nm, Model UVL-56 Blak-Ray Lamp, Ultraviolet Products, Inc., San Gabriel, CA) and concentrated

ammonium hydroxide (NH_4OH) were used to visualize the various bands. The R_f's were calculated by dividing the distance between the origin and the center of the band by the distance between the origin and the solvent front.

Five bands could be distinquished. Band 1, near the origin, (R_f 0.03), Band 2 (R_f 0.24) and Band 4 (R_f 0.62) turned yellow on exposure to concentrated NH_4OH and were dark purple under UV light. These are typical reactions of flavones and are used as indicators of their presence (57). The R_f's of Band 2 and Band 4 were similar to those reported by Reichert (11). He identified Band 2 as vitexin and Band 4 as containing both glucosylvitexin and glucosylorientin. Band 1 was not reported by Reichert. Bands 3 and 5 showed no color change on exposure to NH_4OH. Band 3 fluoresced bright blue under UV light, while Band 5 fluoresced yellow. The R_f value of Band 3 was the same at that for pure ferulic acid .

To separate glucosylorientin from glucosylvitexin, Band 4 was cut out, eluted from the paper with methanol and re-chromatographed as described above using 3:1:1 tertiary butanol:glacial acetic acid:water (TBA) as the solvent. Development time was about 24 hours. As expected, two distinct yellow bands were visible on exposure to concentrated NH_4OH. TBA Band 1 had an R_f of

0.37, which corresponded to the band Reichert (11) identified as glucosylorientin. TBA Band 2 had an R_f of 0.54 which corresponded to the band Reichert identified as glucosylvitexin.

UV SPECTRAL ANALYSIS OF BANDS

Since the R_f's obtained in the paper chromatography of the millet methanol extract differed slightly from those reported by Reichert (11), it was felt that further evidence was needed to identify each band. Flavonoids are usually identified by dissolving them in methanol and then obtaining spectral scans, as well as by the R_f's obtained from paper chromatography with HOAc and with TBA. Mabry et al. (57) describe six different possible scans that can be used in combination to definitely identify a flavonoid. Since Reichert had already identified the flavonoids present in pearl millet, only two spectral scans were used here to confirm our identification, one in methanol, and the second in methanol plus sodium methoxide.

PROCEDURE The procedure of Mabry et al. (55) was used for the spectral analyses of the bands obtained from the paper chromatography. The individual bands were cut out and eluted for about 10 minutes in excess spectral grade methanol. The methanol was then poured

off and filtered through a Whatman No. 1 fluted filter paper. Solutions were concentrated by rotary evaporation and made to a final volume of 10 ml with methanol. This solution was used directly or diluted if necessary. Since methanol elutes some UV-absorbing compounds from the paper (57), a blank was prepared by extracting a blank piece of chromatographic paper from the same chromatogram and equal in size to the band eluted.

The solutions were scanned in a Varian DMS 80 UV-visible, double - beam spectrophotometer (Varian Associates, Palo Alto, CA) at 50 nm/ minute from 220-450 nm.

A sodium methoxide (NaOMe) spectrum was also done. NaOMe changes the pH of the solution, shifting the scan only slightly, but characteristically. Three drops of NaOMe stock solution were added to the methanol solution and the spectrum immediately taken as described above. No NaOMe was added to the blank (57).

The stock solution of sodium methoxide, 1.09 M, was prepared by carefully adding 2.5 g of freshly cut metallic sodium to 100 ml dry spectroscopic grade methanol. The solution was stored in a glass container with a tight- fitting rubber stopper.

QUANTITATION OF FERULIC ACID

Reichert (11) reported that pearl millet contained a high concentration of ferulic acid, about 158 mg/100 g grain. The purpose of Diet VII was to determine the effect of a high dietary concentration of ferulic acid on the thyroid histology and hormone pattern of rats, as well as to measure any other anti-nutrient effects. The following procedure was used to determine the concentration of ferulic acid in all of the diets used in the feeding study and in the methanol extracts used to prepare Diets III, IV and V.

PROCEDURE The high performance liquid chromatography (HPLC) method of Hahn et al. (10) was used to quantitate the amount of ferulic acid in each diet. A Varian Model 5000 high performance liquid chromatograph (HPLC) (Varian Associates, Palo Alto, CA) with a Rheodyne auto-injector (10 microliter sample loop) was used. Detection was by ultraviolet absorption at 254 nm using a Varian Varichrome Model VUV-10 liquid chromatograph UV-visible light detector. Retention times and peak areas were obtained with a Hewlett-Packard HP 3392A integrator (Avondale, PA). An Econosphere C18 column (Alltech Associates, Deerfield,

IL), 250 mm X 4.6 mm, was used. Ferulic acid (Sigma Chemical Co., St. Louis, MO) was dissolved in spectral grade methanol for use as a standard.

A multi-step gradient was used to optimize separation. Two eluants were used: A, methanol-butanol (92:8), and B, water-acetic acid (98:2). The separation was programmed isocratically for 10 minutes at 95% solvent B, followed by a 7.5 minute linear gradient to 85% solvent B. This was continued isocratically for 13.5 minutes, then followed by a 10- minute linear gradient to 50% solvent B.

SAMPLES This procedure was used to determine the amount of ferulic acid in samples of the methanol extract used to prepare Diets III, IV and V, as described in the Feeding Study.

The following exhaustive extraction procedure was used to determine the amount of free and bound ferulic acid in each diet.

Samples from each prepared diet were defatted by a 12-hour Soxhlet hexane extraction and allowed to air dry. To determine the amount of free ferulic acid, 25 g of the defatted grain was stirred with a magnetic stirrer for 72 hours in 75 ml methanol in a covered beaker. At 24-hour intervals the methanol was removed by separation in a Buchner funnel, using Whatman No. 4 filter paper, and the grain was returned to the beaker

with fresh methanol. The methanol extracts were combined and the final solution was reduced to about 1/5 its original volume in a rotary evaporator, then diluted to 50 ml with methanol. The samples were stored at -20°C to precipitate some sugars (11) and then filtered through a 0.45 micron pore size filter before HPLC injection. This extract was analyzed for free ferulic acid content. The grain was reserved for extraction of bound ferulic acid.

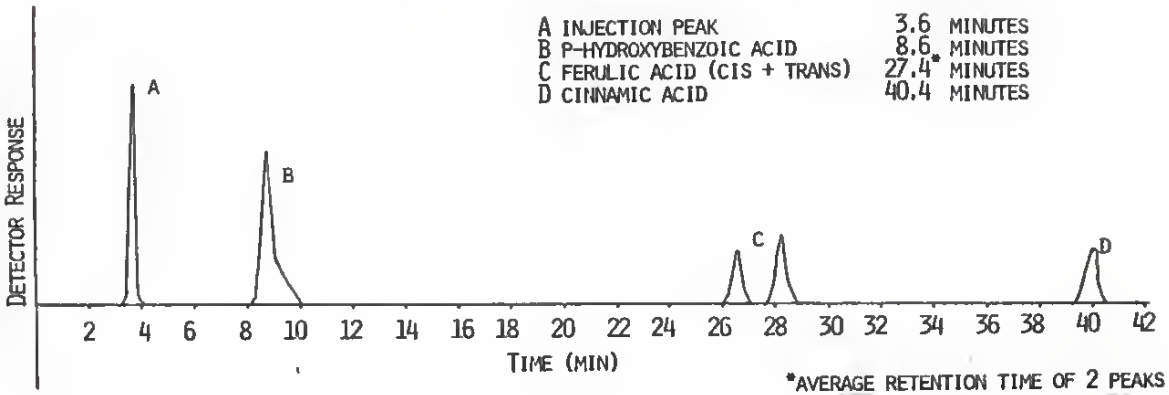
A modified procedure of Hahn et al. (17) was used to extract bound ferulic acid. Five grams of the residual grain from the free ferulic acid extraction procedure described above was hydrolyzed for 1 hour with 20 ml of 2 N hydrochloric acid (HCl) in a boiling water bath. The hydrolysate was cooled to room temperature, and the solids were separated in a Buchner funnel using Whatman No. 4 filter paper. Both the liquid and the solid residue were extracted twice with 20 ml of ethyl acetate and the extracts were pooled. The pooled extracts were evaporated to dryness on a rotary evaporator and then dissolved in 35 ml methanol. These solutions were filtered through a 0.45 micron pore size filter before analysis for bound ferulic acid.

Figure 1 shows the HPLC chromatogram obtained using this procedure from the standard containing 0.01mg/ ml ferulic acid and 0.005 mg/ml each of p-hydroxybenzoic and cinnamic acids. Less p-hydroxybenzoic acid and cinnamic acid was used in the standard, since they were found to have a greater absorbance than ferulic acid at 254 nm. The p-hydroxybenzoic acid and the cinnamic acid were added for qualitative identification of these phenolic acids which are known to exist in both sorghum (17) and pearl millet (16). The lower limits of detection using this method were found to be 0.002 mg ferulic acid/ ml. Ferulic acid elutes as two peaks, cis and trans forms, with an average retention time of 27.4 min. The more polar p-hydroxybenzoic acid is eluted earlier (retention time = 8.6 min), whereas cinnamic acid, which is less polar, elutes later at approximately 40.4 min.

Figures 2-5 show the chromatograms for the free and bound phenolic acids of both sorghum and pearl millet obtained by using this method. In general, the retention times are slightly higher for the compounds in the extracts than for the standards. The chromatograms of the bound phenolic acids of both sorghum and pearl millet (Fig. 4 and 5) show only one peak for ferulic acid. These results are similar to those of Hahn et al. (17), but no explanation is given in that paper as to

FIGURE 1. HPLC CHROMATOGRAM OF PHENOLIC STANDARDS

	RETENTION TIME
A INJECTION PEAK	3.6 MINUTES
B P-HYDROXYBENZOIC ACID	8.6 MINUTES
C FERULIC ACID (CIS + TRANS)	27.4* MINUTES
D CINNAMIC ACID	40.4 MINUTES



*AVERAGE RETENTION TIME OF 2 PEAKS

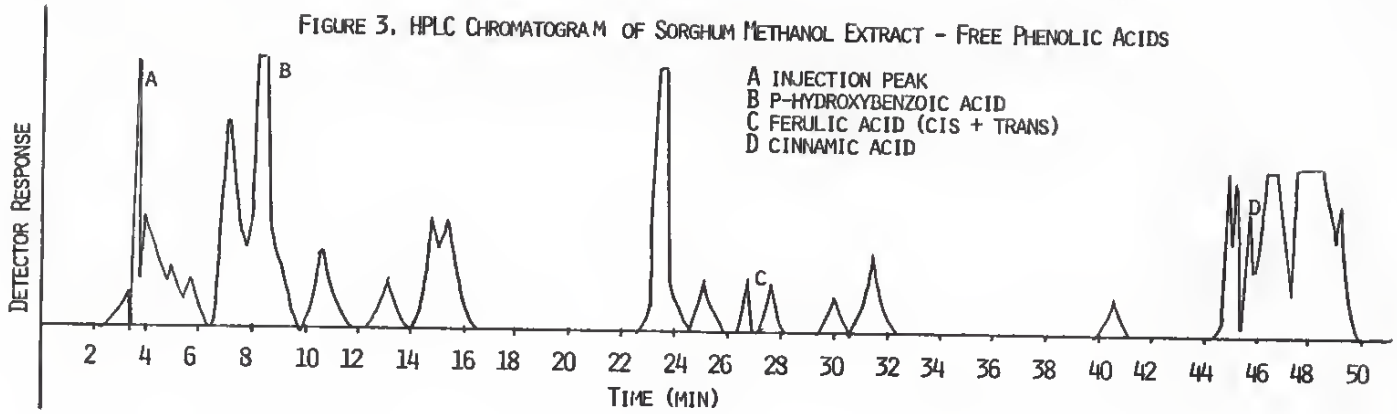


FIGURE 3. HPLC CHROMATOGRAM OF SORGHUM METHANOL EXTRACT - FREE PHENOLIC ACIDS

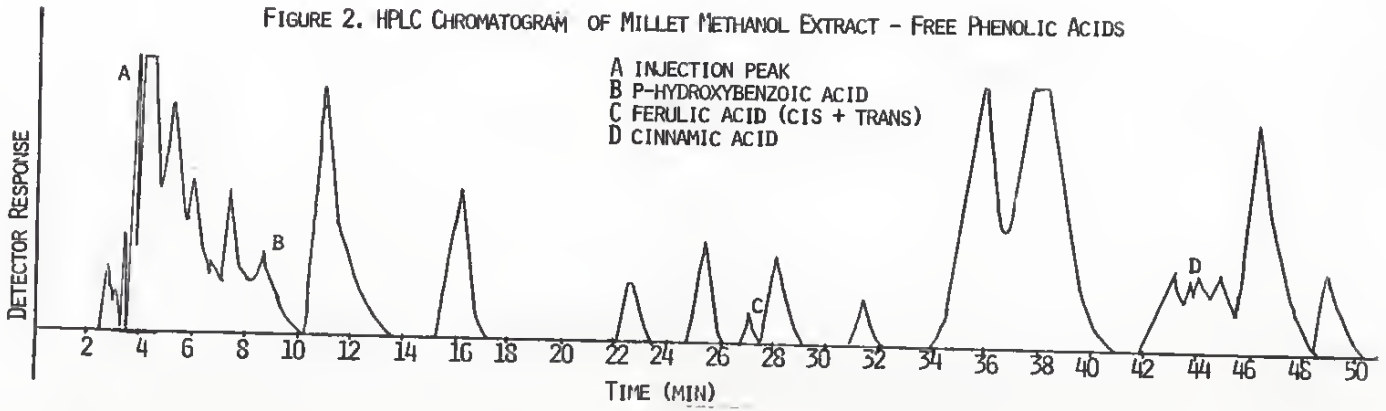


FIGURE 2. HPLC CHROMATOGRAM OF MILLET METHANOL EXTRACT - FREE PHENOLIC ACIDS

FIGURE 4. HPLC CHROMATOGRAM OF MILLET METHANOL EXTRACT - BOUND PHENOLIC ACIDS

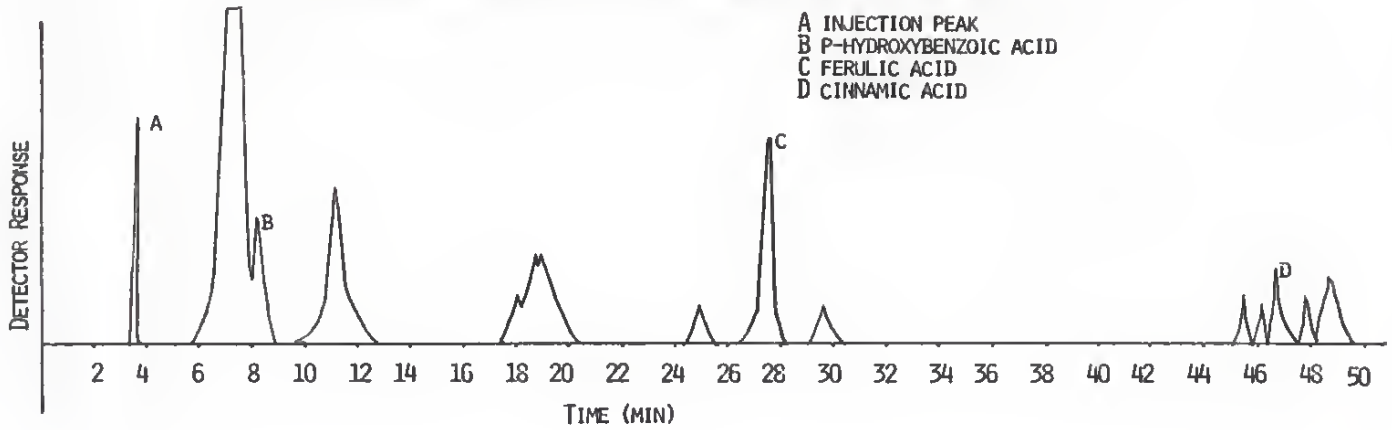
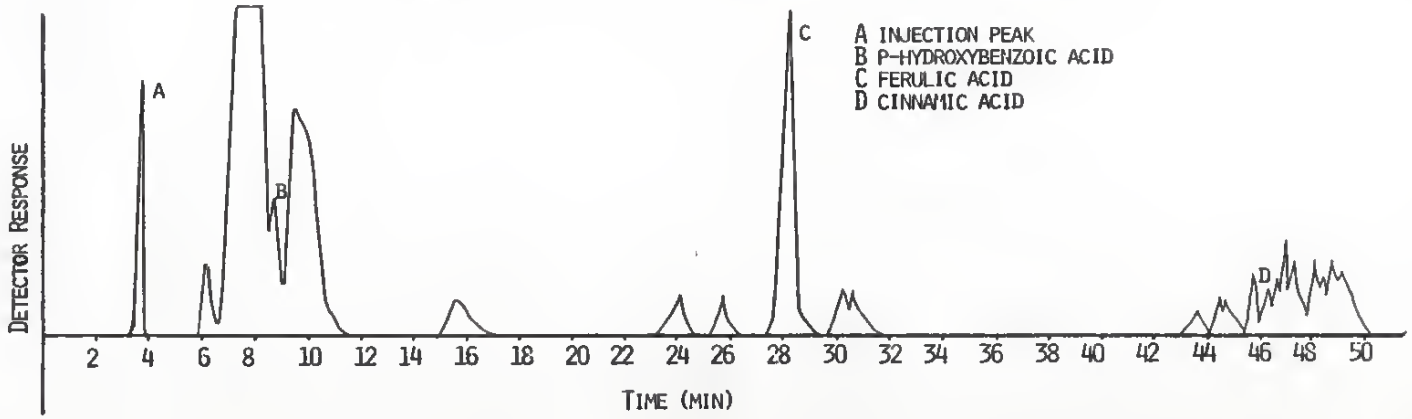


FIGURE 5. HPLC CHROMATOGRAM OF SORGHUM METHANOL EXTRACT- BOUND PHENOLIC ACIDS



why one of the ferulic acid peaks dissappears. It is assumed that the acid hydrolysis used to release the bound phenolic acids changes all ferulic acid to one form.

HPLC PROCEDURE FOR QUANTITATING FLAVONOID CONTENT

Since the diets in the feeding study were designed, in part, to determine the effect of the C-glucosylflavones found in pearl millet on the thyroid histology and thyroid hormones, as well as on the growth rates of rats, the following method was developed to separate and quantitate the flavones in a quick, efficient manner. Though a number of procedures have recently been developed to separate various flavonoids by HPLC, no procedure had as yet been developed to separate the recently identified flavones glucosylvitexin and glucosylorientin. Most separation and identification of flavonoids is still done by paper chromatography, a rather tedious method. Reichert (11) used a complicated spectrophotometric procedure and calculations to quantitate the total flavone concentration in pearl millet methanol extracts, but the results obtained depend on using the same extraction procedure he described. Since a different extraction procedure was used in this study, it was felt that a

quicker, more efficient method could be developed using HPLC.

PROCEDURE The Varian liquid chromatograph and detector, the Econosphere C-18 column, and the Hewlett-Packard integrator used in the ferulic acid determinations were also used to determine the concentration of flavonoids in the diets. Flavonoid standards were dissolved in methanol. Purified glucosylvitexin, glucosylorientin and vitexin were a gift of Dr. Robert Reichert, Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada. Purified vitexin was a gift of Dr. Thomas Mabry, University of Texas, Austin, Texas.

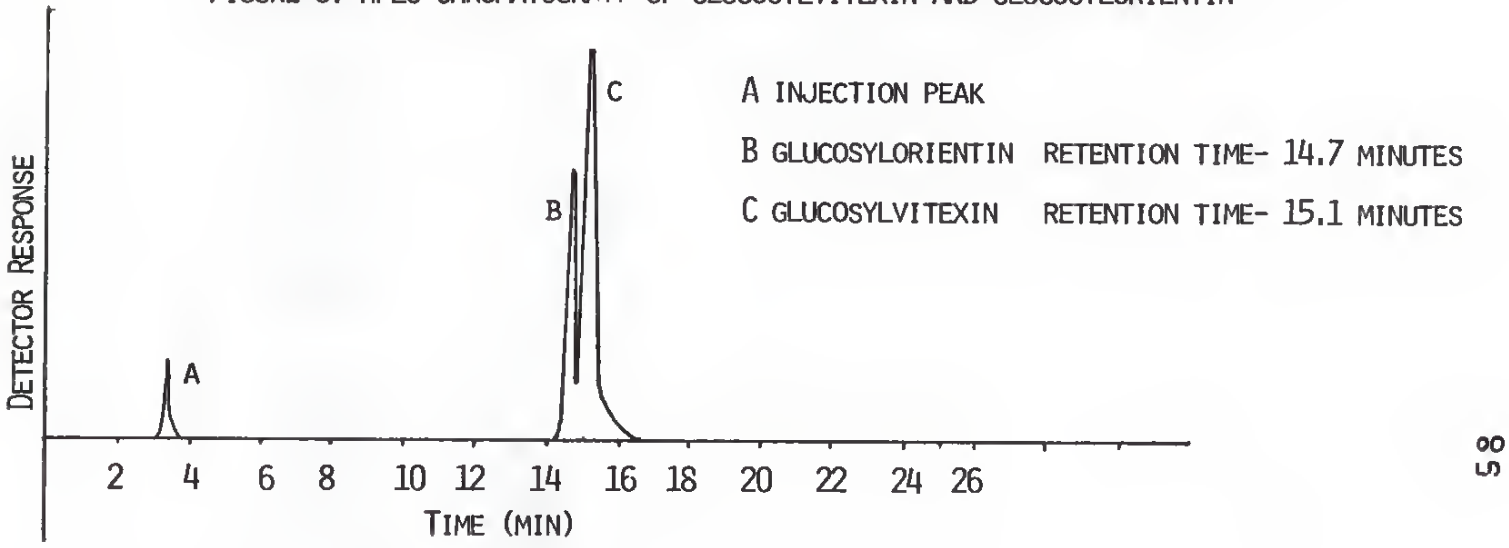
The following program was developed to separate the pearl millet flavonoids. Two solvents were used: A, acetonitrile and B, water-formic acid (19:1). The separation was programmed isocratically for 4 minutes at 90% solvent B, followed by a linear gradient for 6 minutes to 80% solvent B which was programmed isocratically for 2 minutes, followed by a 15 -minute linear gradient to 0% B. The ultraviolet detector was set at 340 nm.

All flavonoid concentrations are reported as glucosylvitexin equivalents. The percent of the total flavonoid area on the chromatogram due to glucosylorientin was calculated.

Figure 6 shows the chromatogram for glucosylorientin (retention time, 14.7 min) and glucosylvitexin (retention time, 15.1). Figure 7 shows the chromatogram obtained for vitexin (retention time, 14.9 min). Figure 8 is the chromatogram of pearl millet methanol extract, showing only two peaks, glucosylorientin, and glucosylvitexin plus vitexin. These last two could not be separated in this system. The retention times of the flavones in the extract were slightly higher than in the standards. The wetted millet extracts were found to have only one peak, with a retention time the same as that found for glucosylvitexin (15.1 min). Figure 9 is the chromatogram of the sorghum methanol extracts. No peaks are found in the region where the C-glucsoylflavones were found to elute.

SAMPLES Samples of the methanol extracts used to prepare Diets III, IV and V were analyzed by this procedure as well as the methanol extracts prepared from each diet in the 72-hour extraction procedure for free ferulic acid described above. These extracts were stored at -20^{o}C and then filtered through a 0.45 micron pore size filter before analysis. The eluted bands from the paper chromatography of the methanol extracts were also chromatographed as standards.

FIGURE 6. HPLC CHROMATOGRAM OF GLUCOSYLVITEXIN AND GLUCOSYLORIENTIN



FLAVONE STANDARD

FIGURE 7. HPLC CHROMATOGRAM OF VITEXIN STANDARD

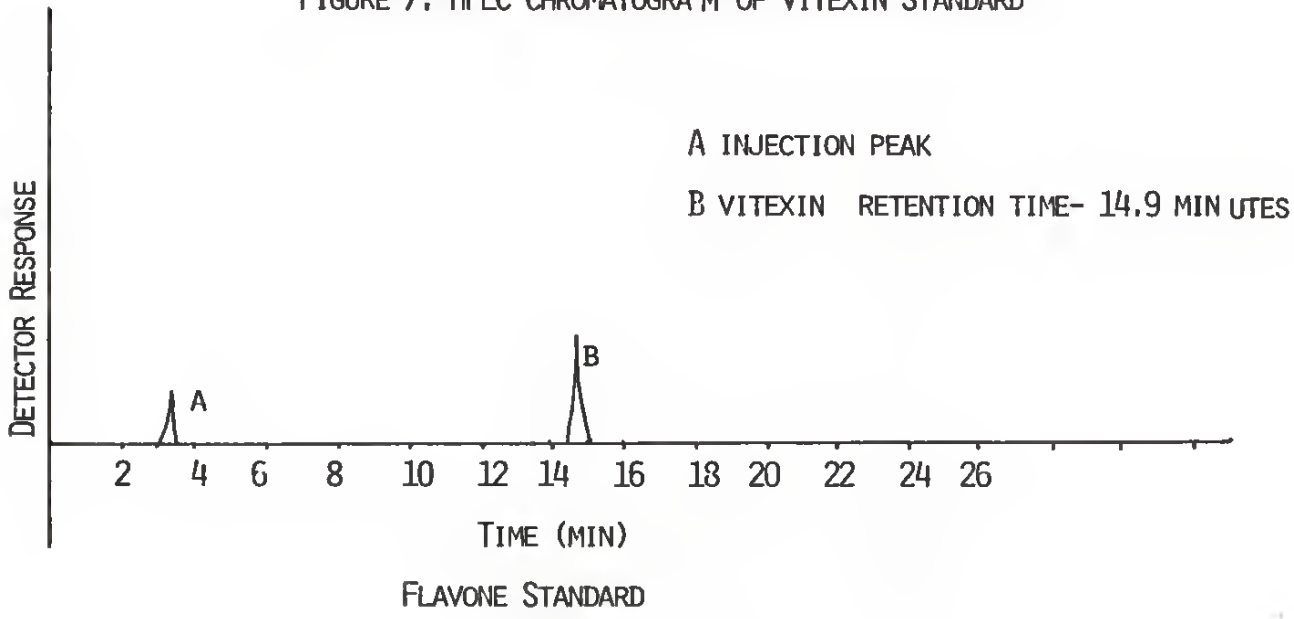
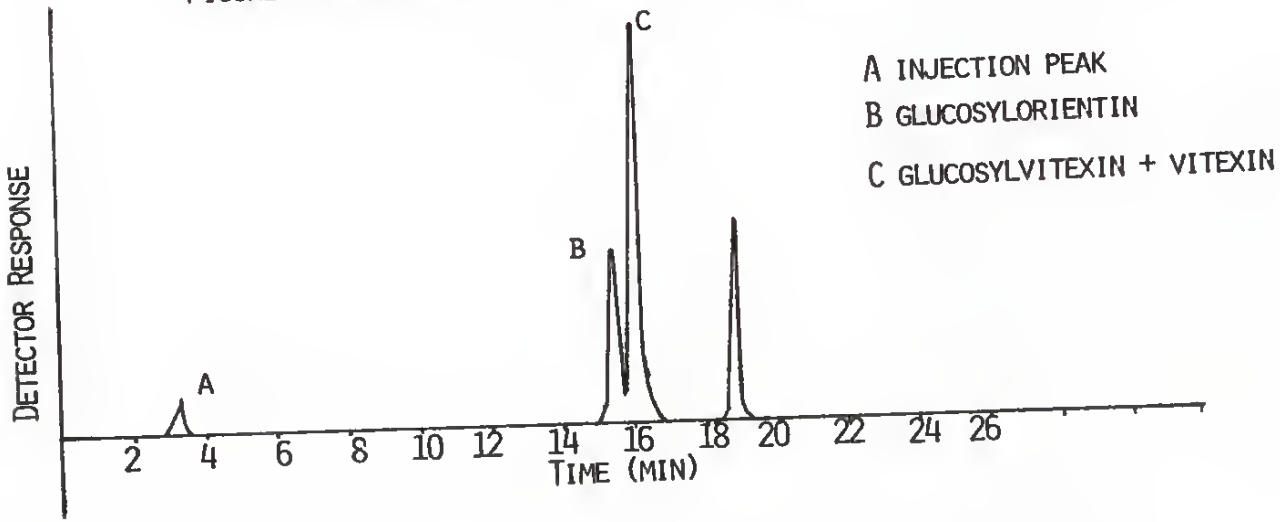


FIGURE 8. HPLC CHROMATOGRAM OF MILLET METHANOL EXTRACT- FLAVONOIDS



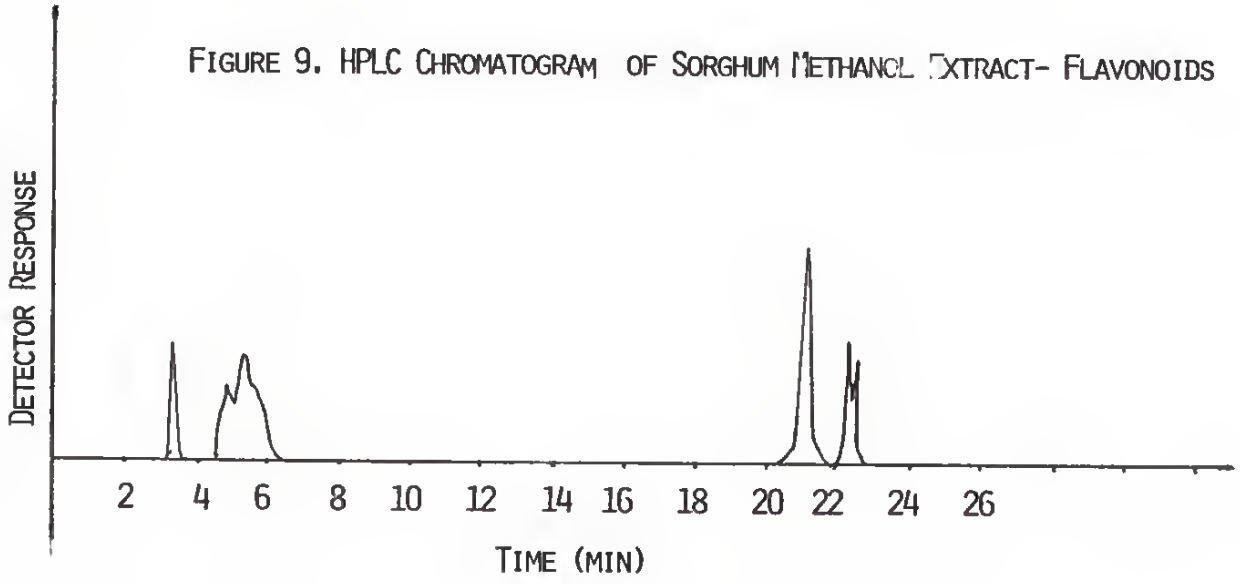


FIGURE 9. HPLC CHROMATOGRAM OF SORGHUM METHANOL EXTRACT- FLAVONOIDS

TREATMENT OF PEARL MILLET

Pearl millet was subjected to a number of treatments in order to study the stability of the flavonoid component under different processing conditions. Most of the treatments were chosen to simulate common methods of preparing millet and sorghum porridges. Autoclaving was done because previous work by Klopfenstein et al. (7) indicated that severe heat treatment of the pearl millet before feeding it to rats alleviated the goitrogenic effects.

GRAIN A sample of the ground pearl millet used to prepare the diets for the feeding study was used.

TREATMENTS Pearl millet was defatted by a 12-hour Soxhlet hexane extraction and allowed to air dry.

Treatment 1. Lactic Acid, pH 3

Lactic acid was added to distilled water until pH 3 was reached. Fifty milliliters of that solution were stirred into 15 g of pearl millet and the mixture held in a beaker, covered, at room temperature for 24 hours. The grain was then uncovered and allowed to dry at room temperature with frequent stirring. Drying time was approximately 24 hours.

Treatment 2. Lactic acid, pH 3, boiled

Fifty milliliters of the same lactic acid used in

Treatment 1 was added to 15 g of grain. The mixture was boiled for 5 minutes, then allowed to air dry.

Treatment 3. Potassium hydroxide (KOH), pH 11

Potassium hydroxide was added to distilled water until pH 11 was reached. Fifty milliliters was then added to 15 g pearl millet and the mixture treated as described in Treatment 1.

Treatment 4. Potassium hydroxide, pH 11, boiled

Fifty milliliters of the KOH prepared in Treatment 3 was added to 15 g pearl millet, and then the mixture was treated as in Treatment 2.

Treatment 5. Distilled water, boiled

Fifty milliliters of distilled water was added to 15 g of pearl millet and the mixture then treated as described in Treatment 2.

Treatment 6. Autoclaved

Fifteen grams of pearl millet was autoclaved at 15 psi for 20 minutes.

EXTRACTION PROCEDURE After treatment, 30 ml of methanol was added to 15 g of the dried grain, and the mixture was stirred with a magnetic stirrer for 1 hour. The grain and methanol were separated in a Buchner funnel with Whatman No. 4 filter paper and the procedure repeated with fresh methanol. The methanol extracts were combined and then reduced on a rotary evaporator to

15 ml. The extract was retained at -20°C for at least 24 hours to precipitate some sugars (11), then filtered through a 0.45 micron pore size filter before being analyzed by HPLC for flavonoid content as previously described.

CHEMICALS All chemicals used throughout this work were reagent grade and obtained from Fisher Scientific (St. Louis, MO) unless otherwise specified. Spectral grade solvents for spectral scans and HPLC analyses were obtained from Fisher Scientific. Double- distilled water was used in all HPLC analyses.

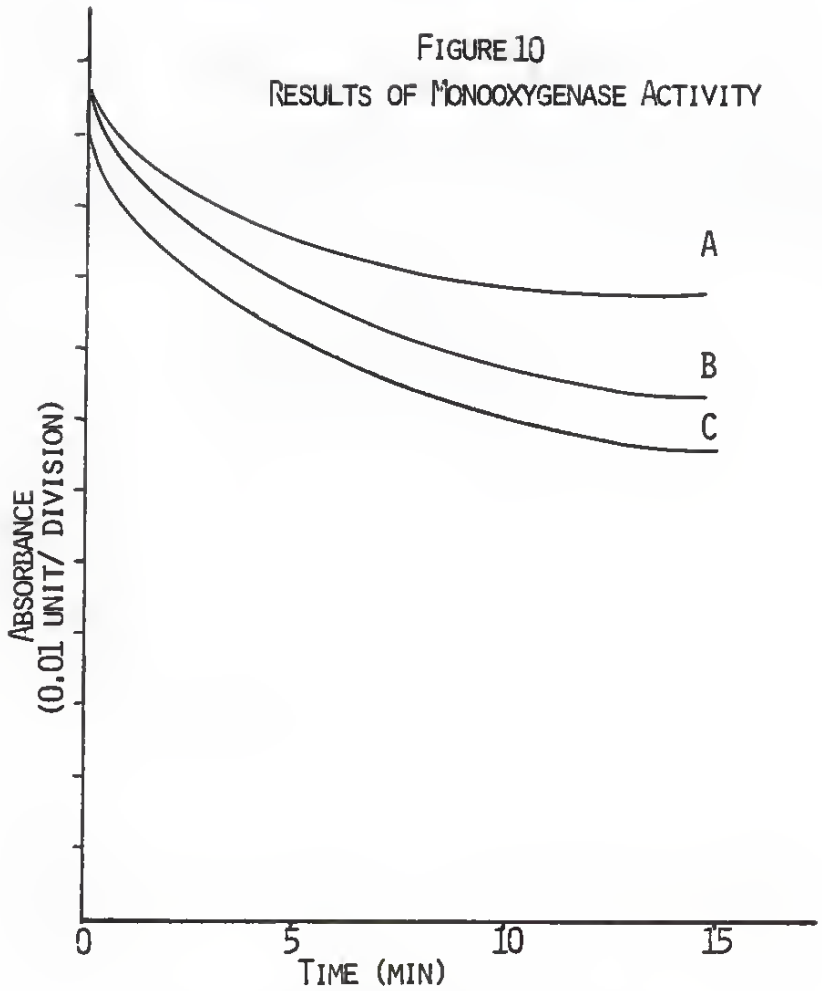
RESULTS AND DISCUSSION

Results of Monooxygenase Assay

The results of the monooxygenase assay were inconlusive. Initial assays showed that the pearl millet extracts seemed to have somewhat greater activity than the sorghum extracts (Fig. 10). It was felt that the low activity might be the result of enzyme inhibition by polar lipids remaining in the extract (54), or due to low concentrations of any active compounds. Assays using extracts three times as concentrated as the initial extracts did not show activity significantly greater than that found in the initial assays, and increasing the concentration of the enzyme added to these extracts did not increase the activity observed. Extracts from chloroform-extracted samples also showed activity only equal to the initial assays. In fact, no extract prepared showed any greater activity than that found in the initial extracts.

When the pearl millet extract was added to an assay mixture containing methimazole, the enzyme showed initial activity, but activity quickly dropped,

FIGURE 10
RESULTS OF MONOOXYGENASE ACTIVITY



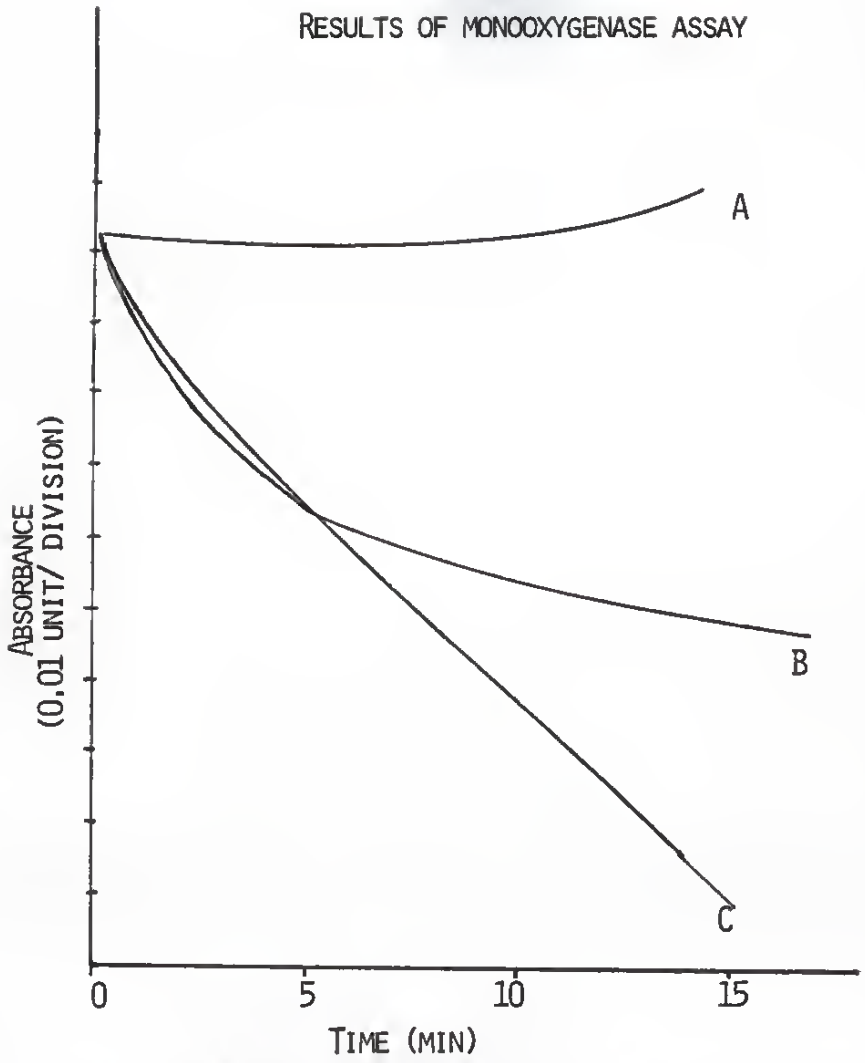
A = MILLET BUFFER EXTRACT - ENZYME
B = SORGHUM BUFFER EXTRACT + ENZYME
C = MILLET BUFFER EXTRACT + ENZYME

indicating that the enzyme was inhibited by the extract (Fig. 11).

Analysis of all results at this point led to the conclusion that it would not be possible to use the monooxygenase assay as a tool to show the presence of nucleophilic sulfur-containing compounds in phosphate buffer extracts of pearl millet. There are several explanations for the negative results observed. The concentration of any active compound may have been too low to allow for the observation of any significant activity. Also, it is possible that no procedure used succeeded in removing all enzyme-inhibiting short chain fatty acids. It is also possible that other non-lipid inhibitors were present in the extract. It has been noted that some flavones inhibit microsomal monooxygenases *in vitro*, though they may induce activity *in vivo* (58). The effect of flavonoids on microsomal enzyme activity has not been well studied, and the effects of glucosylvitexin and glucosylorientin, which have been recently identified, are not known at all at this time. However, flavonoids have frequently been found to inhibit *in vitro* enzyme activity (58), and it is possible that they acted as inhibitors in the assay used here.

FIGURE 11

RESULTS OF MONOOXYGENASE ASSAY



A = METHIMAZOLE STANDARD - ENZYME

B = METHIMAZOLE STANDARD + ENZYME + MILLET BUFFER EXTRACT

C = METHIMAZOLE STANDARD + ENZYME

RESULTS OF NITROPRUSSIDE AND FERRIC NITRATE TESTS

The results of the nitroprusside test for sulfur-containing compounds and the ferric nitrate test for thiocyanates were negative for all extracts tested, except where thiocyanate had been added as an internal standard (Table 3). When thiocyanate was added as an internal standard, the results were positive for the ferric nitrate test only. No different results were obtained when trichloroacetic acid was added to the extracts to precipitate protein (55). Sodium hydroxide was also added to some samples tested since it is known that this will release thiocyanate bound to other chemicals such as p-hydroxybenzoate (55). The addition of NaOH to the extracts did not effect the results obtained.

The results indicate that the pearl millet tested here probably does not contain thiocyanate or isothiocyanate. Klopfenstein et al. (7) reached a similar conclusion based on the results of feeding studies using pearl millet supplemented with iodine. Generally, the goitrogenic effects of thiocyanate-induced goiter can be overcome by supplemental dietary iodine, whereas those of millet-induced goiter cannot.

The results of the nitroprusside test, which is a more general test for sulfur-containing compounds, seem to indicate that no large concentrations of sulfur-

TABLE 3

RESULTS OF NITROPRUSSIDE AND FERRIC NITRATE TESTS FOR SULFUR-CONTAINING COMPOUNDS

	Nitroprusside	Ferric Nitrate
SAMPLES		
Buffer standard	-	-
Pearl millet, Extraction Procedure A*	-	-
Pearl millet, Extraction Procedure C*	-	-
Pearl millet, Water extract **	-	-
Pearl millet, Phosphate Buffer Extract**	-	-
Pearl millet, Acetate Buffer Extract**	-	-
Pearl millet, Water Extract + Thiocyanate standard**	-	dark red
Pearl millet Phosphate Buffer Ext.+ Thiocyanate standard**	-	dark red
Pearl millet, Acetate Buffer Ext + Thiocyanate standard**	-	dark red

*See Extraction Procedure for Monooxygenase activity page 27

**See Nitroprusside Test Extraction Procedure page 30

containing compounds are present in the extracts of the pearl millet tested in this study, but the results obtained when thiocyanate was added to the extracts as an internal standard indicated that the test is not very sensitive. The fact that these same extracts gave positive results when tested with ferric nitrate indicates that the thiocyanate was not destroyed or lost in the extraction procedure. Positive results were obtained with the standards used, indicating that the test solution was viable. Therefore, it is unlikely that any significant concentrations of sulfur-containing compounds were present in these extracts, though some may exist.

Osman et al. (5) reported that a spot isolated from pearl millet extract by paper chromatography showed a positive response to the nitroprusside test. It is possible that further separation of the extracts used here may have given the same result. It is also possible that varietal differences or differences in environmental growth conditions might account for the conflicting results.

RESULTS OF THE TEST FOR CYANIDE

Some plants contain compounds that release cyanide when processed or ingested (27). The cyanide is detoxified in the body by a process which results in the

formation of thiocyanate. Since a similar compound may act as the goitrogen in pearl millet, a test for cyanide was performed.

The results of the test for cyanide were positive for pearl millet, but negative for sorghum grain. These results indicate that the pearl millet tested in this study contained a cyanide-containing compound. Such compounds are common in plants, often occuring as cyanogenic glycosides (27). Casava contains the cyanogenic glycoside linamarin, which is known to induce endemic goiter in areas where casava is a staple. Sorghum is known to contain a different cyanogenic glycoside, dhurrin. This compound is found in the leaves, especially in young plants. It is not found in the grain, and so it is usually not a problem forpeople who depend on sorghum as a staple.

Osman et al. (5), using a different method, reported that no cyanide was present in pearl millet, but the method used was not as sensitive as the one used here. The reaction obtained in this study was slight, though obvious, indicating a low concentration of cyanide or cyanide-producing compounds in the pearl millet. The symptoms of goiter resulting from cyanide can generally be alleviated by dietary iodine supplements (27) but the goitrous symptoms resulting

from pearl millet consumption are not (7). Cyanide, therefore, is probably not the only cause of pearl millet-induced goiter.

The fact that pearl millet does contain cyanide may explain the observation made by Osman et al. (5) that the concentration of thiocyanate in the blood of people who consume large amounts of pearl millet was significantly higher than that found in people who ate less pearl millet. Cyanide is detoxified in the body by a reaction that produces thiocyanate (27). Osman et al. (5) had suggested that the high blood thiocyanate concentrations might be the result of an unidentified thioamide in the millet. It is also possible that the cyanide test used here identified the same compound that Osman et al. had identified using the nitroprusside test. Thiocyanates can degrade to cyanide on the addition of acid.

RESULTS OF THE SPECTRAL SCANS OF BUFFER EXTRACTS

The spectral scans of the buffer extracts of pearl millet did not match exactly with any of the standards tested (Table 4). Scans of pearl millet extract containing added indole and thiocyanate were distinctly different from those of pearl millet extract alone, indicating that these two compounds were not present in

TABLE 4

SPECTRAL SCANS OF BUFFER EXTRACTS
ABSORBANCE MAXIMA (nm)

STANDARD	
Methimazole	250
Indole	270, 213
Thiocyanate	200, 220shift
Thiourea	237, 200
Dextrose	199
SAMPLE	
Pearl millet, Extraction procedure C*	276, 210shift, 199
Sorghum, Extraction procedure C*	198
Pearl Millet, Extraction Procedure A*	267, 193

*See Extraction Procedures for Monoxygenase Activity, page 30

the buffer extracts of pearl millet tested in this study.

Scans of sorghum extracts were quite different than those of pearl millet, they showed no absorbance peaks in the 270 nm area, as pearl millet did.

Many known goitrogens contain sulfur atoms (59), yet no clear evidence for a sulfur-containing compound in pearl millet could be found. Astwood (59) had discovered that a number of phenolic compounds, especially those containing the aniline group (NH_2-C_6H_4-), could cause goiter when fed to rats. Aromatic compounds have absorption maxima around 270, as did the pearl millet buffer extracts (Table 4). Fawcett and Kirkwood (16) showed that many aromatic compounds containing electron- donating groups could act as goitrogens, including phloroglucinol (1, 3, 5-trihydroxybenzene) and resorcinol (1, 3-dihydroxybenzene). Also, polyphenolic compounds were implicated as goitrogens in peanuts (28, 29, 30) and in soybeans (32, 33). Reichert (11) identified three polyphenolic pigments in pearl millet. These are the flavones glucosylvitexin, glucosylorientin and vitexin. Though flavonoids are common in many plants, including grains, these particular flavones have thus far been found only in pearl millet. Recently, Reddy (16) identified these same flavones as the precursors of

compounds that produce an unpleasant odor in pearl millet after it has been wetted and then dried.

RESULTS OF PAPER CHROMATOGRAPHY AND UV SCANS OF BANDS

Paper chromatography of the pearl millet methanol extracts gave results very similar to those reported by Reichert (11). The R_f's obtained in this study were slightly higher than those reported by Reichert, probably because of small differences in conditions. The fact that the chromatography paper was cut to fit a slightly smaller chromatocab and differences in temperature, solvents, extraction and application techniques all might account for the small differences observed in the R_f's. The differences were observed with both 15% glacial acetic acid (HOAc) and 1:1:3 tertiary butanol : glacial acetic acid : water (TBA).

The bands obtained by paper chromatography were eluted in methanol and spectral scans were obtained to further characterize the compounds present. The spectra obtained from the bands with R_f's corresponding to glucosylorientin, glucosylvitexin and vitexin matched those reported by Reichert (11) for those flavones.

The paper chromatography of the extract used in this study showed a band at R_f 0.03 (HOAc) that turned

yellow on exposure to concentrated NH_4OH and fluoresced purple under UV light, reactions typically used to identify flavones (57). However, this band was not reported by Reichert. The spectral scan of this band showed absorbances at 335 and 267 nm in methanol and at 395, 342, and 276 nm when sodium methoxide was added. These absorption maxima are characteristic of flavones (57). Mabry et al. (57) have purified many flavones and have reported the R_f's obtained in HOAc and TBA, and the absorption maxima obtained in a number of different solvents. Though these scans reported above are very similar to the ones reported for apiginin, the aglycone of vitexin and glucosylvitexin, the R_f for the unknown is smaller than that reported for apigenin (0.11 in HOAc). It is possible that the pearl millet used in this study contains another, as yet unidentified flavone, in addition to glucosylvitexin, glucosylorientin and vitexin.

RESULTS OF FERULIC ACID QUANTITATION

Exhaustive extraction of the diets used in this feeding study indicated that the pearl millet contained about twice as much total ferulic acid as the sorghum used (Table 5). The amount of free ferulic acid in both pearl millet and sorghum was very similar, but pearl

millet contained more bound ferulic acid.

Diet VI contained less bound ferulic acid and less total ferulic acid than Diet II, but the amount of free ferulic acid was higher. Analysis of the methanol extracts used for diet preparation showed that only about 1/4 of the free ferulic acid (about 0.39 mg/ 100 g) was removed by that extraction procedure. Diet VII was found to contain about ten times more ferulic acid than it was calculated to contain, based on the amount of pure ferulic acid added (280 mg/ 100 g). It was observed that the ferulic acid was in a rather course form when added to the diet, and so was not mixed homogeneously throughout the diet. Since the sample was taken at the end of the feeding study, it is possible that, over time, some of these particles settled to the bottom, increasing the concentration of ferulic acid in this sample.

Since all the diets were formulated from the same two grain samples, it would be expected that similar amounts of bound ferulic acid would be present in all the millet diets and in all the sorghum diets. In discussing the extraction efficiency of their method, Hahn et al. (17) noted that a flocculant precipitate was present at the interface of the ethyl acetate and water which made it difficult to partition the two phases cleanly. It was noted that some of the phenolics may

TABLE 5

FERULIC ACID CONTENT OF DIETS

DIET	AMOUNT FERULIC ACID (mg/100g) Free	Bound	Total
I. Sorghum control	1.2	11.4	12.6
II. Millet control	1.2	23.8	25.0
III. Sorghum + 1X millet extract	1.2	8.4	9.6
IV. Sorghum + 2X millet extract	1.5	7.7	9.2
V. Sorghum + wetted millet extract	1.4	8.8	10.2
VI. Ext'ed millet	2.5	18.2	20.7
VII. Ext'ed millet + ferulic acid	3800	25.2	3825.2

have been caught in the precipitate, introducing a source of error into the method. The precipitate was observed here with both the pearl millet and the sorghum extracts and this may explain some of the variation obtained in measuring the bound ferulic acid.

The sorghum used in this study was found to have a greater total ferulic acid content than that reported by Hahn et al. (17). In testing seven different varieties, they found total ferulic acid contents ranging between 1 mg/ 100 g and 3.4 mg / 100 g. The higher concentration found in the sorghum used in this study may be a result of varietal differences, environmental differences in growth conditions or small differences in the extraction procedure. The total ferulic acid found in the pearl millet used here (25 mg/ 100 g) was significantly lower than the amount reported by Reichert (11), which was 158 mg / 100 g. This may be due to differences in extraction procedures. Reichert used alkali to hydrolyze the bound ferulic acid, whereas the procedure employed here used acid. Hahn et al. (17) stated that the efficiency of the acid extraction procedure was lower for ferulic acid than for other phenolic acids, probably due to destruction of the ferulic acid during hydrolysis. The differences between the amount of ferulic in the pearl millet used in this

study and that reported by Reichert for pearl millet could also be a result of varietal differences or differences in growth conditions.

RESULTS OF FLAVONE QUANTITATION

The total amount of C-glucosylflavones (expressed as glucosylvitexin units) found in the pearl millet used in this study was 122 mg/ 100 g grain (Table 6). This was essentially the same as that reported by Reichert (11) (124 mg/ 100 g). In this study, it was found that about 28.5% of the total flavone content was due to glucosylorientin, the other 71.5% was due to glucosylvitexin and glucosylorientin. This is also very close Reichert's report of 66% glucosylvitexin, 25% glucosylorientin and 9% vitexin. Reichert used paper chromatography and a spectrophotometric method to determine the percent absorbance due to the total C-glucosylflavones. Results obtained using that procedure depended on the extraction method used. The HPLC method used in this study was developed as a faster, less empirical, method of analyzing the methanol extracts of pearl millet for glucosylvitexin, glucosylorientin and vitexin.

It was found that, in preparing the diets, only about 1/4 of the total flavone content was removed by

analyzed by HPLC, it was found to contain only one flavone peak. The retention time of the peak indicated that it was from glucosylvitexin or vitexin; no glucosylorientin was observed. Paper chromatography of the wetted millet extract indicated that glucosylvitexin was also absent; only vitexin was present. HPLC analysis of the wetted millet extract for free phenolic acids showed a very different profile from that obtained for the methanol extract of the untreated pearl millet (Fig. 12). An increase in the amount of p-hydroxybenzoic acid in the wetted millet extract was especially apparent, as was the appearance of a number of new compounds. Further HPLC analysis of the wetted millet extract indicated that two of the peaks had the same retention times as resorcinol and ploroglucinol. These compounds eluted very early in the program (retention times = 4.9 minutes, phloroglucinol, 6.0 minutes, resorcinol) and are part of the large peak observed in the wet millet extract at about 3 - 7 minutes (Fig. 12). Examination of the chromatograms indicates that there is an increase in the compounds eluting at this time in the methanol extract after the millet is wetted. The changes observed in the chromatograms of the wetted millet extract, as compared to those of the dry millet extract, indicate that the

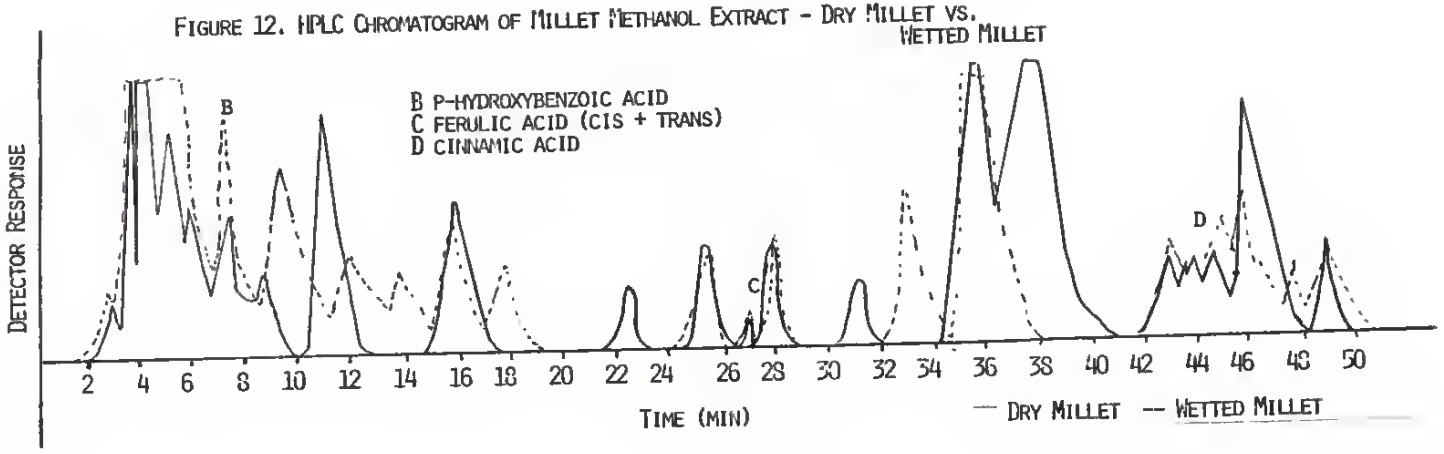
TABLE 6

C-GLUCOSYLFLAVONE CONCENTRATIONS

DIET	AMOUNT FLAVONE (mg/100 g grain)*
I. Sorghum control	0
II. Millet control	122
III. Sorghum + 1X millet extract	33
IV. Sorghum + 2X millet extract	66
V. Sorghum + wetted millet extract	32
VI. Ext'ed millet	91
VII. Ext'ed millet + ferulic acid	133

*expressed as glucosylvitexin units

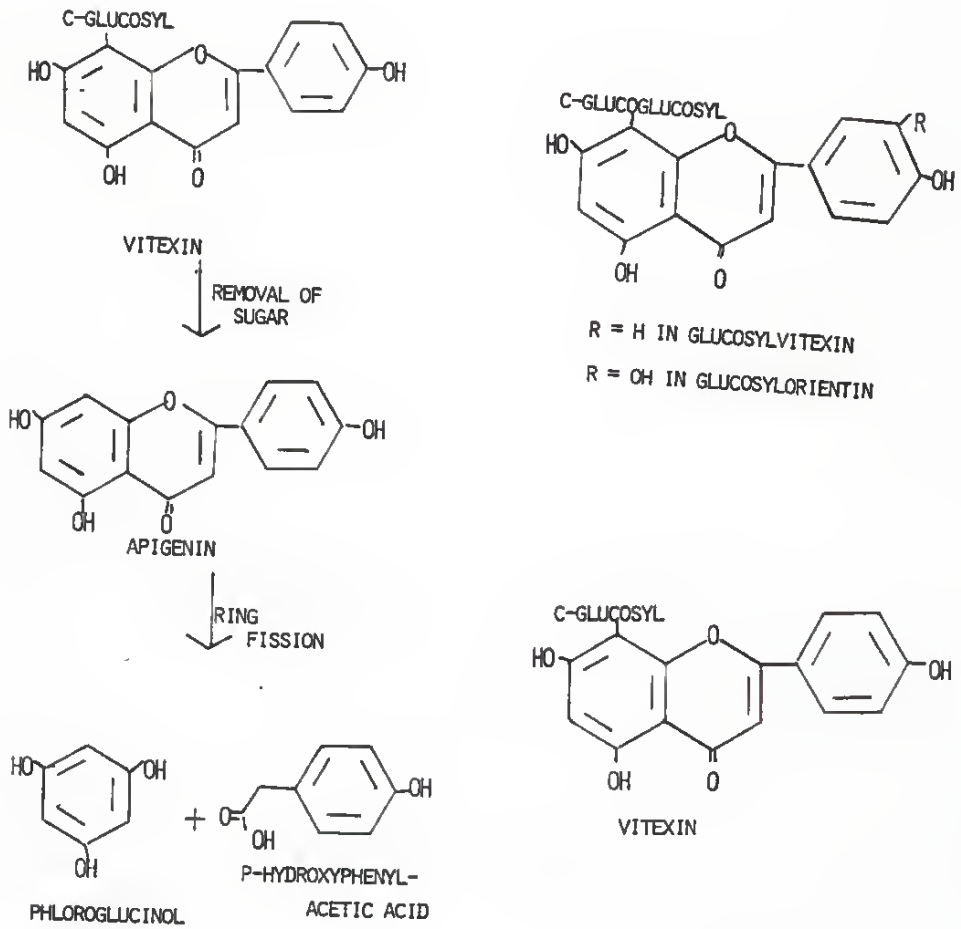
FIGURE 12. HPLC CHROMATOGRAM OF MILLET METHANOL EXTRACT - DRY MILLET VS. WETTED MILLET



amount and type of flavonoids and phenolic compounds changed as a result of wetting the millet. The reason for this change is not known, but wetting the millet may initiate some enzyme action which catalyzes the transformation. The fact that it occurs is not suprising. Reddy (16) showed that the C-glycosylflavones were the precursors of some odor-causing compounds produced when pearl millet is wetted, indicating that some change in these compounds occurs when the millet is wetted. It has been found that the flavones are broken down by the intestinal microflora after ingestion, forming benzoic acids and hydroxyphenols such as phloroglucinol or resorcinol (Fig. 13) (47). Further study of the chemical changes occurring when pearl millet is wetted is needed before these changes are fully understood.

Figure 13

Ring Fission of C-glucosylflavones in Pearl Millet



RESULTS OF THE FEEDING STUDY

Nutritional Effects

In this study, the rats fed pearl millet diets showed significantly greater weight gains than those fed sorghum diets (Fig. 14). Feed efficiencies were also higher for pearl millet diets than for sorghum diets (Fig. 15). All the diets were isocaloric but percent protein was higher in the pearl millet diets (15%), than in the sorghum diets (9%). Also, it has been shown a number of times (6, 7, 8, 9) that pearl millet has a higher protein efficiency ratio (PER) than sorghum. This has been attributed, in part, to the greater digestibility of pearl millet. An amino acid imbalance has also been noted in sorghum grain. Its high leucine content may interfere with the utilization of valine and isoleucine.

The addition of the millet methanol extract to the sorghum further lowered the feed efficiencies of those diets (Fig 15). The weight gains found with Diets III, IV, and V were lower than for Diet I (sorghum control) (Fig. 14). Diet IV (sorghum plus 2X millet extract) had twice as much C-glucosylflavones (66 mg /100 g) Diet III (sorghum plus 1X millet extract, 32 mg/ 100 g). The extracted pearl millet diets (Diets VI and VII) produced

FIGURE 14

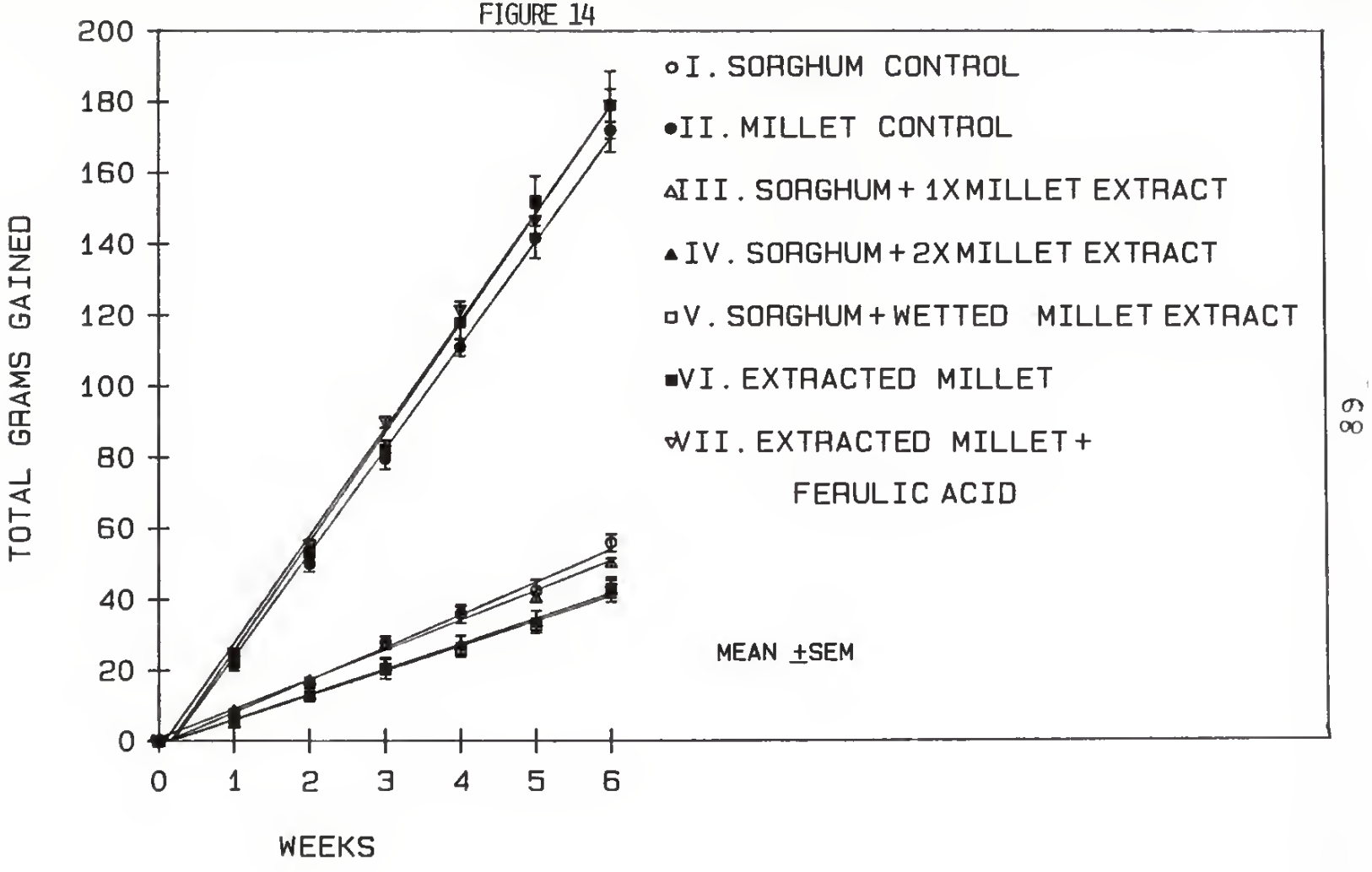
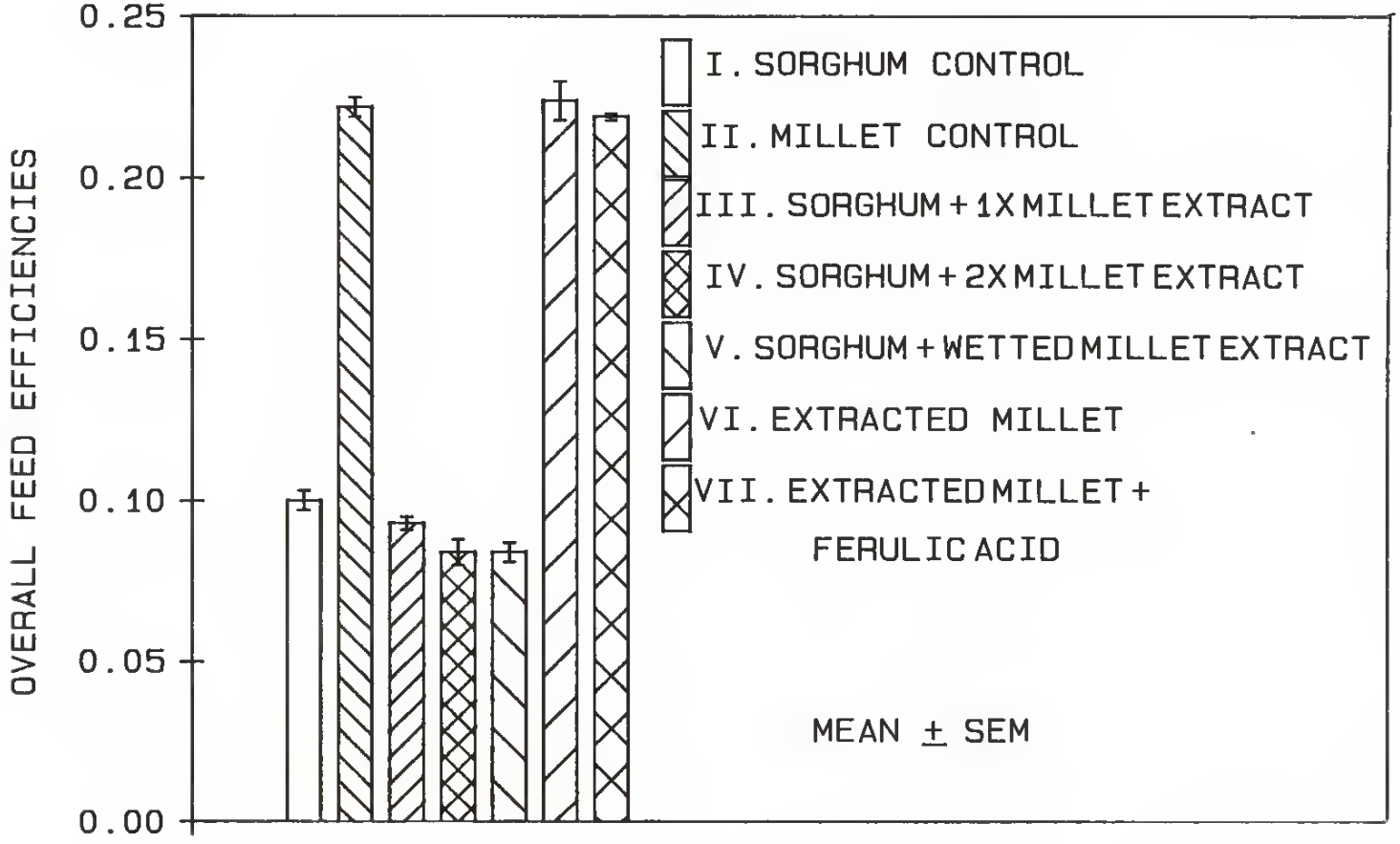

TOTAL GRAMS GAINED
200
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
WEEKS
I. SORGHUM CONTROL
II. MILLET CONTROL
III. SORGHUM + 1X MILLET EXTRACT
IV. SORGHUM + 2X MILLET EXTRACT
V. SORGHUM + WETTED MILLET EXTRACT
VI. EXTRACTED MILLET
VII. EXTRACTED MILLET + FERULIC ACID
MEAN ±SEM

FIGURE 15


OVERALL FEED EFFICIENCIES
0.25
0.20
0.15
0.10
0.05
0.00
I. SORGHUM CONTROL
II. MILLET CONTROL
III. SORGHUM + 1X MILLET EXTRACT
IV. SORGHUM + 2X MILLET EXTRACT
V. SORGHUM + WETTED MILLET EXTRACT
VI. EXTRACTED MILLET
VII. EXTRACTED MILLET + FERULIC ACID
MEAN ± SEM

slightly, though not significantly, higher weight gains and feed efficiencies than the unextracted millet diet. Diet VII contained a very large amount of added ferulic acid which apparently had no effect on feed efficiency or weight gain.

It would seem that some component of the pearl millet methanol extract had an anti-nutritional effect, but it was apparently not ferulic acid or the flavones. If it were the flavones, it would be reasonable that Diet IV (sorghum + 2X millet extract) would have a greater anti-nutritional effect than Diets III (sorghum + 1X millet extract) and V (sorghum + wetted millet extract). Though Diet IV did have a greater anti-nutritional effect than Diet III, it did not have a greater effect than Diet V. The ferulic acid concentrations were the same in all three. Also, adding unusually large amounts of ferulic acid to Diet VII, a pearl millet diet, did not reduce the feed efficiency. This would also indicate that ferulic acid did not act as an anti-nutrient in this study.

Wetting the millet before methanol extraction (Diet V) had the same effect on feed efficiencies and weight gains as adding twice as much extract from dry millet (Diet IV). As noted above, wetting the millet changed the type of flavones present and the amount and type of phenolic compounds in the methanol extract. This would

suggest that some some change occurs when the millet is wetted that may increase the anti-nutrient activity.

Little has been written about the nutritional effects of phenolic acids and flavonoids. Reichert et al. (42) conducted a feeding study using high-tannin sorghum, low-tannin sorghum and pearl millet. They concluded that any anti-nutrient effects of the phenolic compounds in pearl millet were about equal to those observed in low-tannin sorghum. Kuhnau (47) concluded that flavonoids are "semi-essential nutrients" for mammals and calls them "vitamin-like" in activity. Hahn et al. (41), in discussing the phenolic compounds of sorghum, state that phenols apparently have no anti-nutrient properties. On the other hand, it is known that phenolic acids and flavones are toxic to some organisms, showing bacteriostatic, virostatic and fungistatic effects (41, 47). It is also possible that flavonoids, and perhaps other smaller phenolic compounds, may bind to proteins, decreasing their digestibility. It is known that flavonoids can have an inhibitory effect on enzyme activity <u>in vitro</u> (58). Also, it is known that condensed tannins, which are essentially flavonoid polymers, can bind to and precipitate protein, making it unavailable nutritionally (41). It seems likely that flavonoids and other

phenolic compounds might also bind to proteins. The nutritional effects may not be as great with these smaller compounds as they are with tannins, but in high enough concentrations, they may act as anti-nutrients. The results of this feeding study indicate that the effects of the flavonoids, glucosylvitexin, glucosylorientin, and vitexin and ferulic acid on feed efficiencies and rat growth rates are not nutritionally significant at the concentrations tested. The pearl millet methonal extracts of both dry and wetted millet do however, appear to have antinutrient effects.

Thyroid Hormone and Histology Results

Rats fed pearl millet diets had distinctly different thyroid hormone patterns when compared to those fed sorghum. Millet-fed rats showed significantly lower triiodothyronine (T_3) and thyroxine (T_4) concentrations than sorghum-fed rats (Table 7). This pattern is somewhat different than those observed previously by Klopfenstein et al. (6, 7, 8, 9). In those studies, low T_3 concentrations were observed in millet-fed rats, but T_4 concentrations were found to be higher in millet-fed rats than in sorghum-fed rats.

In past studies, it was theorized that the hormonal differences might be explained by the presence of a

TABLE 7

DIET	T_3[+] (ng/ml)	T_4[+] (ng/ml)
I. Sorghum control	2.43a*	86.2a
II. Millet control	1.45b	67.9b
III. Sorghum + 1X millet extract	2.58a	82.6a
IV. Sorghum + 2X millet extract	2.68a	84.9a
V. Sorghum + wetted millet extract	2.54a	87.4a
VI. Ext'ed millet	1.40b	60.7b
VII. Ext'ed millet + ferulic acid	1.46b	62.0b

[+] T_3 = Triiodothyronine, T_4 = Thyroxine
*Means followed by the same letter are not significantly different at $p < 0.05$.

chemical which inhibits conversion of T_4 to T_3. If such were the case, it would be expected that the ratio of T_3:T_4 would be lower in pearl millet-fed rats than in sorghum-fed rats. In this study, as in the studies of Klopfenstein et al. (6, 7, 8, 9) this was found to be true. The ratio of T_3:T_4 was signficantly lower than that found in sorghum fed rats (Fig. 16). In rats fed Diet VI (extracted pearl millet) the ratio was somewhat higher than in rats fed Diet I (untreated pearl millet) suggesting that some active compound may have been removed. In rats fed Diet V, sorghum + wet millet extract, the ratio was somewhat lower than in the other sorghum diets, suggesting that some active compound may have been added.

Microscopic examination of thyroid sections of rats used in this study showed that definite goiter-like changesoccurred in the thyroids of all rats fed pearl millet diets. These changes include enlarged thyroid colloid follicles (especially in the periphery of the gland), flattened epithelial cells, uneven staining colloid and evidence of hyperplasia (Fig. 17). These results agree with those found by Klopfenstein et al. (6, 7, 8, 9) who have reported similar changes in the thyroids of pearl millet- fed rats. There was little or no difference between the thyroids of rats fed Diet VI (extracted pearl millet) and those fed Diet II

FIGURE 16

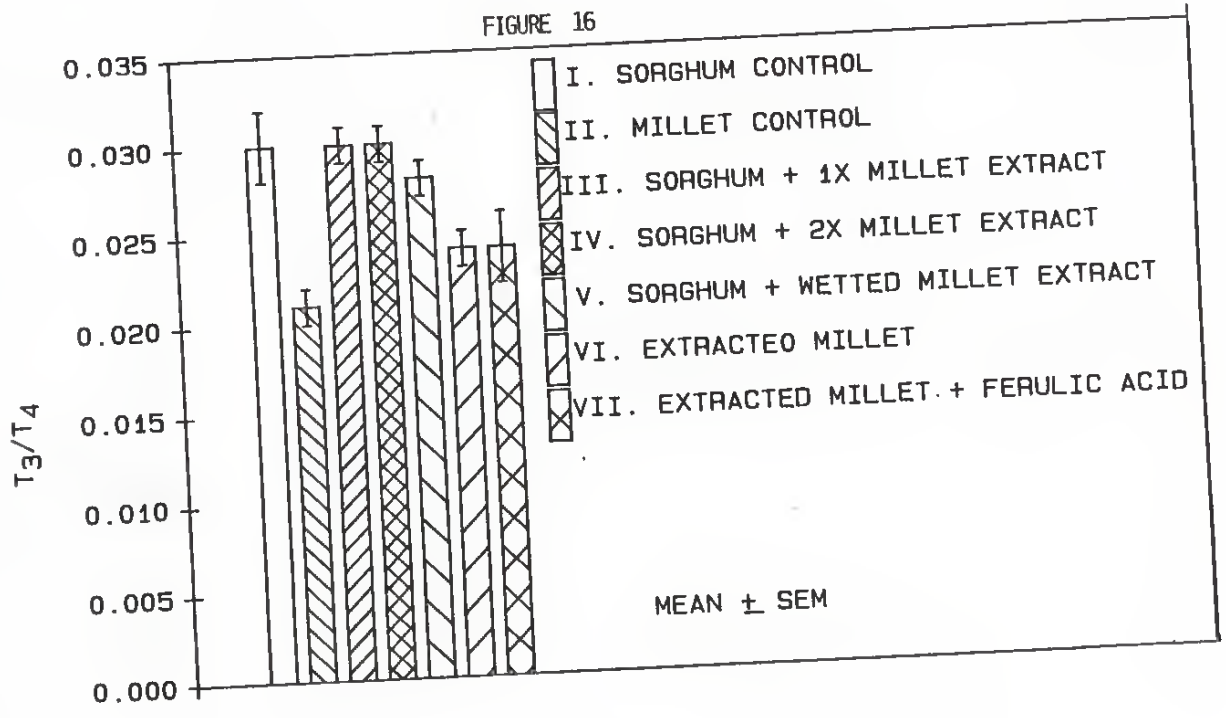

0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
T_3/T_4
I. SORGHUM CONTROL
II. MILLET CONTROL
III. SORGHUM + 1X MILLET EXTRACT
IV. SORGHUM + 2X MILLET EXTRACT
V. SORGHUM + WETTED MILLET EXTRACT
VI. EXTRACTED MILLET
VII. EXTRACTED MILLET + FERULIC ACID
MEAN ± SEM

FIGURE 17

Photomicrographs of Rat Thyroid Tissue

Typical examples of rat thyroid tissue from rats fed each of the diets used in this study.

A) Diet I - Sorghum Control

B) Diet II - Millet Control

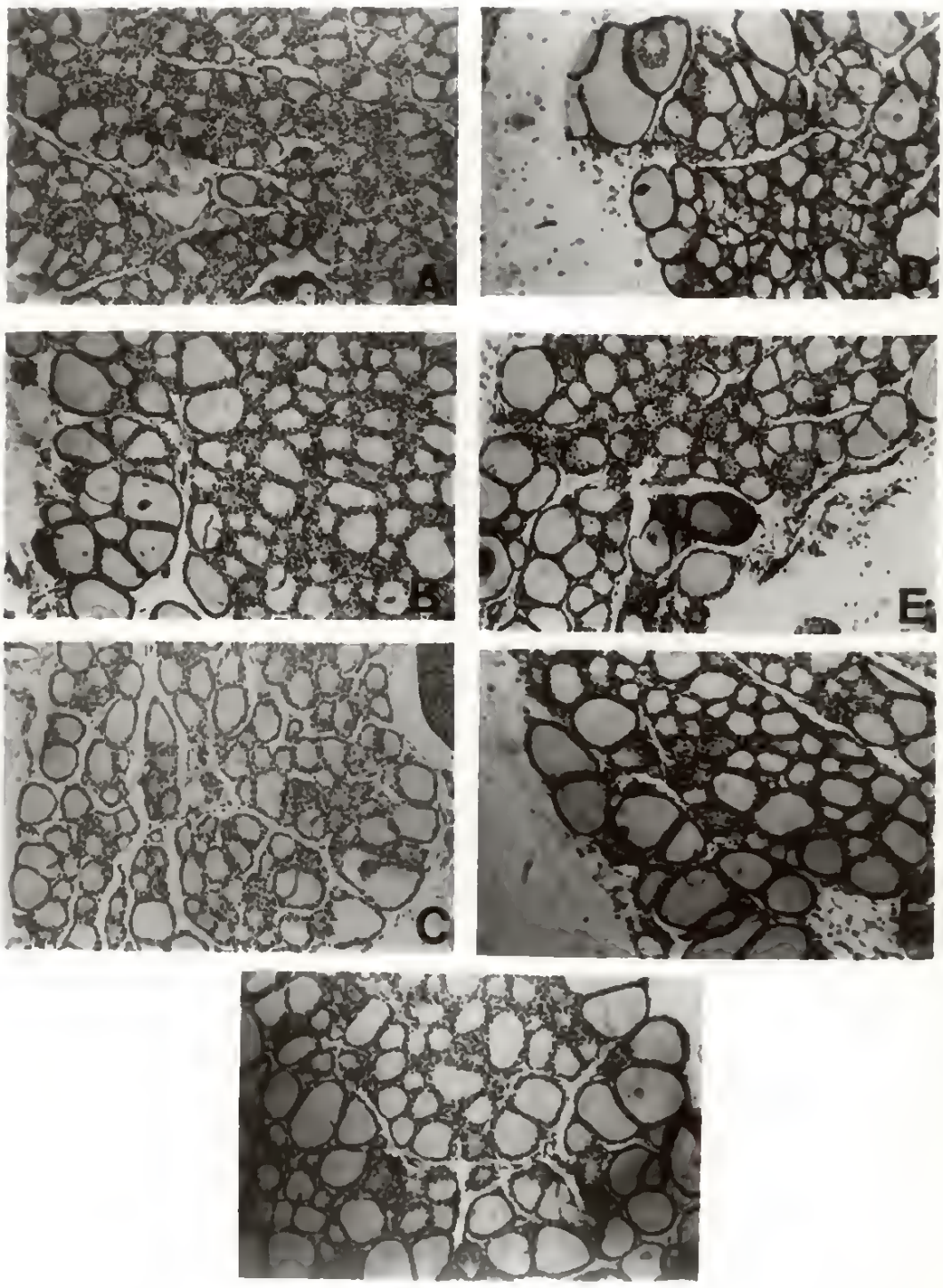
C) Diet III - Sorghum + 1X Millet Extract

D) Diet IV - Sorghum + 2X Millet Extract

E) Diet V - Sorghum + Wetted Millet Extract

F) Diet VI - Extracted Millet

G) Diet VII - Extracted Millet + Ferulic Acid


A
B
C
E

(untreated pearl millet). Considering the apparent inefficiency of the extraction procedure used to prepare the diets, no conclusions can be made from these data about the effects of flavonoids on thyroid histology. Examination of the thyroids from rats fed Diet VII, a millet diet which contained an unusually high concentration of ferulic acid, revealed abnormalities somewhat different from those seen in other millet-fed rats. The thyroid colloid follicles were enlarged, but there was little evidence of hyperplasia and the follicles were thought to be somewhat cuboidal in shape, something not seen in any other group. The effects on the thyroid were described as toxic, but distinctly different from the effects seen in any of the other diets.

The rats fed Diet I (untreated sorghum) showed normal thyroid histology, with uniformly averaged-sized thyroid colloid follicles, even-staining colloid, and no signs of hyperplasia. Rats in Diets III, IV, and V all showed signs of thyroid abnormalities typical of those seen in pearl millet-fed rats, especially enlarged follicles and hyperplasia (Fig. 18). It was not possible to distinquish any differences in the severity of the thyroid abnormalities in the thyroids of rats fed Diets III, IV, and V (the sorghum + millet extract diets). It was also not possible to distinguish between

FIGURE 18

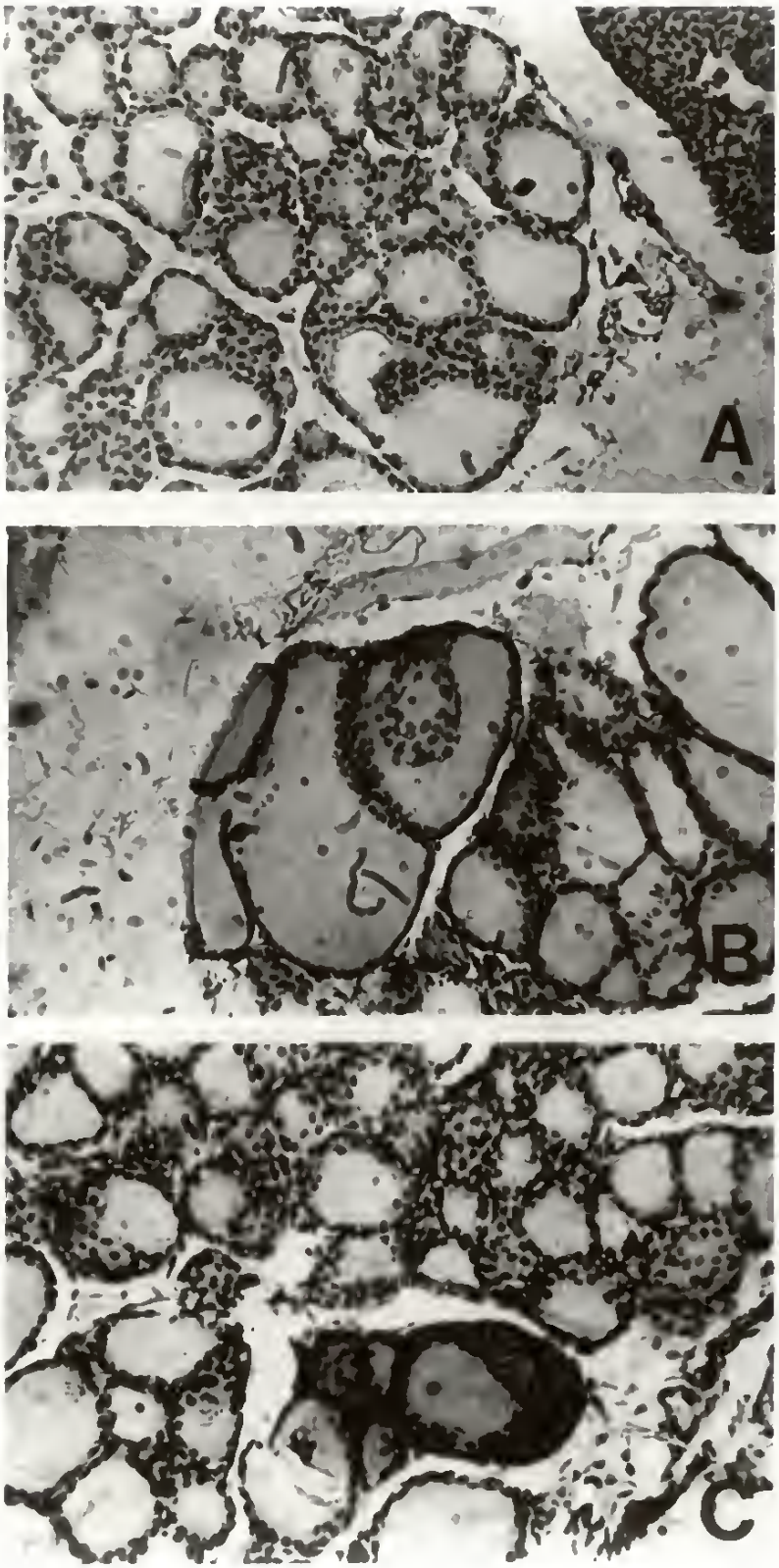
Photomicrograph of Rat Thyroid Tissue Showing Severe Hyperplasia

These photomicrographs are of the same tissue shown in Fig. 17, taken at a higher magnification.

A) Diet III - Sorghum + 1X Millet Extract
Note infolding of epithelial cells into large colloid follicle

B) Diet IV - Sorghum + 2X Millet Extract
Note infolding of epithelial cells into large colloid follicle, flattening of epithelial cells

C) Diet V - Sorghum + Wetted Millet Extract
Note hyperplasia of large colloid follicle


A
B
C

the rats fed sorghum plus millet extract diets and those fed Diet I (millet control) or Diet VI (extracted millet). This was true despite the fact that only about 1/4 the concentration of flavones was present in the sorghum plus millet extract diets as compared to the millet control. These diets also contained a much lower flavone concentration (about 1/3 as much) than the extracted millet diet. It is possible that the flavones, or products formed from the flavones, are very strong goitrogens. It is also possible that the cyanide-producing compound found to exist in the pearl millet may be important in the histological changes observed in the rats fed sorghum plus millet extract diets.

The thyroid histology and thyroid hormone changes found in animals fed sorghum + pearl millet methanol extract (Diets III, IV and V) indicate that a goitrogen definitely was extracted from the pearl millet in the methanol. The methanol extracts were not completely characterized, but they are known to contain a number of phenolic compounds. Many phenolic compounds have been identified as goitrogens. A flavonoid in peanut skins (28, 29, 30) and another in soybeans (32, 33) have been implicated as goitrogens. Fawcett and Kirkwood (23) recognized p-hydroxybenzoic acid, phloroglucinol and resorcinol as goitrogens, the last two of which are

especially active. It was previously noted that, in this study, the methanol extract of wetted pearl millet showed a large increase in p-hydrobenzoic acid. It was also noted that resorcinol and phloroglucinol may have been formed when the millet was wetted. When that extract was added to sorghum (Diet V), it resulted in lower T_3:T_4 ratios for those animals than for animals fed Diet III (sorghum plus 1X millet extract). Also, since it is known that flavones are digested by gut bacteria to form phloroglucinol and other phenolic compounds, the flavonoids in the extract may form goitrogens after digestion (47).

The evidence gathered in this feeding study would seem to indicate that the flavonoids, or products formed from them when the millet is wetted or ingested, are likely candidates for the millet goitrogen. The cyanide-producing compound may also be involved in the goitrogenicity of pearl millet. On the other hand, too little is known about all the components of the millet methanol extract to completly understand the goitrogenicity of pearl millet. Most plants contain phenolic acid and flavonoids. Sorghum contains the phenolic acids found in pearl millet, including p-hydroxybenzoic acid, which is known to be a goitrogen. Its concentration has not been measured in pearl millet.

In fact, sorghum seems to actually contain a greater number of phenolic acids, though it is difficult to say at this point, since many unidentified compounds exist in the methanol extracts of both sorghum and pearl millet. Sorghum and other grains contain flavonoids, though not all contain flavones.

Flavonoids are extremely common food components, so it would seem that if they are goitrogens in pearl millet, then almost all foods would have goitrogenic properties. On the other hand, the flavonoids are a very large class of compounds and, though they are all basically similar in structure, their chemical and biological activities vary greatly depending on small structural differences such as the degree and site of hydroxylation, the degree of methylation, and the degree, site and type of glycosylation (47). Some flavonoids are much more readily degraded by intestinal microflora than others and the resulting products vary depending on the structure of the original compound (51).

Still apiginen, which is the aglycone of glucosylvitexin and vitexin, is found in many plants, either as the aglycone or as other C-glycosides or glycosides. At the present time, not much is known about the concentration of flavonoids in various plant parts. Though many flavonoids have been identified in

many plants, little quantitative work has been done. Flavonoids seem to be most common in flowers, stems, leaves or outer layers of fruits or seeds, parts of plants frequently removed before eating or not considered food. It may be that pearl millet grain is unusual in that it contains a high concentration of flavonoids. Reichert (11) reported that most of the flavonoids are concentrated in the millet bran, but millet is such a small grain the bran is seldom removed in traditional processing. Also, millet is a staple in parts of the world where few other foods are available. Therefore, people may be forced to eat a large amount of pearl millet to survive, and, as a result, they may ingest an unusually large amount of the flavonoids, increasing the goitrogenic effect. Since wetting the millet appears to increase its goitrogenicity, suggesting enzyme activity, pearl millet may contain an unique or particularly active enzyme that produces large quantities of goitrogenic compounds from the flavonoids. Typical methods of preparing pearl millet include wetting, then drying it, which may further increase the goitrogenic effect.

At the present time then, some evidence seems to point to the C-glycosylflavones, glucosylorientin, glucosylvitexin and vitexin, as the goitrogenic agents

in pearl millet. Wetting the millet before methanol extraction seems to enhance the goitrogenic activity of the extract. Since the glucosylvitexin and the glucosylorientin were destroyed and the phenolic compounds present in the millet changed as a result of this treatment, it is possible that the flavones themselves are not goitrogens but that some phenolic compound or compounds (such as p-hydroxybenzoic acid, resorcinol or phloroglucinol) formed during their degradation may actually be the goitrogen(s). The cyanide-containing or cyanide-producing compound found in this study may increase the goitrogenicity of the pearl millet. It is also possible that some other component, as yet unidentified, may be responsible for the goitrogenic activity observed.

Exactly how these compounds might act as goitrogens is not clear at this point. Fawcett and Kirkwood (23) suggest that phenolic compounds act by competitively binding iodine, preventing the formation of iodotyrosines. It has been shown in rats fed arachidoside, a flavonoid, that the concentration of iodinated phenols is higher than in control rats (30), supporting this hypothesis. The goitrogenic activity of compounds that competitively bind iodine usually is overcome by dietary iodine supplements. Such is not the case with pearl millet (7). It has also been suggested

that phenolic compounds act by inhibiting iodide peroxidase (22). Klopfenstein et al (6, 7), have suggested, based on thyroid hormone evidence that the pearl millet goitrogen acts by inhibiting formation of T_3 by peripheral deiodination of T_4. Since tyrosine, the amino acid precursor of the thyroid hormones, contains a phenolic group, it is possible that the specific structure of glucosylvitexin or vitexin, or of one of the products formed in the degradation of the pearl millet flavones allows it to act as a competitive inhibitor of some enzyme involved in the production of the thyroid hormones.

RESULTS OF TREATMENTS ON FLAVONOID CONTENT

None of the treatments used appeared to destroy the glucosylvitexin, glucosylorientin or vitexin present in pearl millet (Table 8). The procedure used here to extract the flavones was not exhaustive, so it is not possible to tell if any reduction in flavone concentration in the methaonal extract was due to destruction of the flavones or to a decrease in their ability to be extracted from the pearl millet.

The millet treated with acid was found to have 108 mg flavone/ 100 g grain. This would suggest that the

TABLE 8

C-GLUCOSYLFLAVONE CONCENTRATION IN TREATED MILLET

TREATMENT	mg FLAVONE/ 100 g GRAIN*
1. Soaked in Acid	108.0
2. Boiled in acid	32.8
3. Soaked in Base	57.0
4. Boiled in base	7.6
5. Boiled in water	12.3
6. Autoclaved	48.9
Untreated millet+	27.5
Wetted millet+	26.0

*expressed as C-glucosylvitexin units
+methanol extracts used in preparation of Diets III, IV, and V

acid treatment made the flavones more susceptible to extraction, perhaps by changing their ability to hydrogen bond. It is not suprising that the flavones were not destroyed by acid treatment, since it has been noted that C-glucosylflavones are unique of all the flavonoids in being particullary resistant to acid hydrolysis (38). In HPLC chromatograms of the methanol extracts from all the treated grain, except that of wetted millet extract, it appeared that both glucosylorientin and glucosylvitexin were present in ratios similar to those found in untreated grain.

If the flavones are indeed the goitrogenic agents in pearl millet, it may be possible to remove them by soaking the millet in acid, which may release the flavones so that they can be more easily extracted in water. More work remains to be done before the full effects of these treatments on the amount of flavonoids present and on the goitrogenic activity of the pearl millet are known.

CONCLUSION

It is apparent from the thyroid histological and hormonal data obtained in the feeding study that the goitrogen or a precursor of the goitrogen was extracted by methanol. All animals fed the sorghum diets with pearl millet methanol extract added showed significant goiter-like thyroid histological and/or hormonal changes typical of those seen in animals fed pearl millet. These include abnormal thyroid histological features such as enlargment and hyperplasia of the colloid follicles and, at least in the sorghum diet plus wetted millet extract, a decrease in the T_3/ T_4 ratio similar to that seen in pearl millet-fed animals. Animals fed untreated sorghum did not exhibit these changes, while animals fed pearl millet did. Animals fed unextracted millet also exhibited these abnormalities in thyroid histology and hormone patterns, but the T_3/T_4 in these animals was significantly lower than in those fed untreated millet.

It also seems that an anti-nutrient of some type was extracted by the methanol from the pearl millet. Animals fed sorghum plus 2X millet extract and sorghum

plus wetted millet extract showed lower weight gains and feed efficiencies than animals fed untreated sorghum. Also, animals fed extracted millet seemed to have slightly higher weight gains than those fed untreated millet, although feed efficiencies were not greater. It is not clear from this study whether the anti-nutrient effect and goitrogenic effect are due to the same or different compounds

Evidence from this study indicates that likely candidates for the goitrogen or precursors of goitrogenic phenolic compounds are the flavones, glucosylvitexin, vitexin, and glucosylorientin. Our data seems to indicate that the flavones per se might not be the goitrogens or the anti-nutrients. Animals fed sorghum plus 1X millet extract, which contained 33 mg flavones/100 g grain, did not show any more severe goiter-like symptoms than those fed sorghum plus 2X millet extract, which contained twice as high a concentration of flavones. Animals fed sorghum plus wetted millet extract, which contained 32 mg flavone/ 100 g grain, showed a decrease in their T_3/T_4 ratio. A similar pattern was observed in the weight gains and the feed efficiencies of these animals. All three diets had very similar ferulic acid contents, indicating that this was not a factor in the goitrogenic activity or as an anti-nutrient. Adding large amounts of ferulic acid to

the pearl millet also did not induce any anti-nutrient effect or an increase in the goitrogenic effect. This evidence seems to suggest that neither ferulic acid nor the flavonoids *per se* are responsible for the goitrogenic or the anti-nutrient effects observed as a result of the methanol extract.

It is possible that the flavonoids are a precursor of some compound that acts as a goitrogen or an anti-nutrient. The fact that wetting the millet seems to increase the anti-nutrient effect and also the effect on the thyroid hormones seems to support this idea, especially since both glucosylvitexin and glucosylorientin disappear completely in the process. Also, it was noted that the pattern of free phenolic compounds was altered in these chromatographs. A number of new phenolic compounds seem to appear and the apparent concentrations of others increase. The concentration of p-hydroxybenzoic acid seems to increase and phloroglucinol and resorcinol may be formed. All of these substances are known to act as goitrogens and the last two are particularly active (23). It is probable that the changes occurring when the millet is wetted affect its goitrogenic and anti-nutrient properties.

It would seem, then, that a number of steps must be

taken before this puzzle becomes clearer. The goitrogenic and anti-nutrient effects of the flavonoids could be further studied by separating these compounds, perhaps on preparative cellulose columns, and then using the purified compounds in a feeding study. Also, the changes occurring in the flavonoid and phenolic acid compounds caused by wetting the pearl millet need to be further studied. During food preparation, pearl millet would almost certainly be wetted, yet this process may be enhancing the goitrogenic or anti-nutrient activity of the grain. Also, it will be important to further identify the methanol-soluble components of pearl millet. Only three phenolic acids, ferulic, cinnamic and p-hydroxybenzoic, and three flavones, glucosylvitexin, glucosylorientin and vitexin, have been identified, but there are certainly a number of other phenolic acids and perhaps other flavonoid compounds as well. Other phenolic compounds such as phloroglucinol and resorcinol appear to be present in the wetted millet extract. Further work is needed to positively identify these compounds and quantitate them in the wetted millet and the dry millet.

The HPLC method developed here will aid in further studying and identifying the flavones. It is also helpful to know that the HPLC procedure developed by Hahn et al. (17) for sorghum phenolic acids is

applicable to pearl millet. The procedure developed during this study was found to give results similar to those reported by Reichert (11) who used a spectrophotometric procedure. An advantage to the HPLC procedure is that it gives a better idea as to what flavonoids are present and in what concentration, and it depends less on the extraction procedure used. The HPLC procedure is much easier, faster and more efficient than paper chromatography. It will be useful in studying the changes that occur in pearl millet when it is treated in various ways and in identifying other flavonoids that may be present in the pearl millet. It may also be useful in discovering the fate of the flavonoids once they are ingested by animals. This could be important since the flavonoids may only be the precursors of other compounds which act as goitrogens or anti-nutrients. Breeders, who may be searching for low flavonoid varieties of pearl millet should also find the method useful, because, if the flavonoids are found to be related to the anti-nutrient or goitrogenic effects, low-flavone varieties will be desired.

Obviously much work is left to be done before the goitrogenic effects of pearl millet are fully understood, but this study represents a step closer to the final answer.

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THE FURTHER CHARACTERIZATION OF A GOITROGEN IN PEARL MILLET (*Pennisetum americanum* (L.) Leeke)

by

DIANNE MARIE BIRZER

B. S. University of Illinois, Urbana-Champaign, 1978

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Rat-feeding studies have shown that pearl millet, an important food crop in many parts of the semi-arid tropics, causes symptoms of goiter. The purpose of this study was to further characterize the compounds in pearl millet that are responsible for its goitrogenic activity.

Many known goitrogens are sulfur-containing compounds. A number of procedures, including chemical tests, an enzyme assay, and spectral scans, were employed in an attempt to identify in pearl millet extracts a sulfur-containing compound similar to those known to be goitrogens. No positive results were obtained with any of the procedures used.

It has been found that many phenolic compounds can also act as goitrogens. Recently the flavones glucosylvitexin, glucosylorientin and vitexin have been identified as polyphenolic pigments in pearl millet. A methanol extract containing these substances, as well as other phenolic compounds, was prepared and added to sorghum, which was then fed to rats. Other diets consisted of sorghum plus extract from wetted, then dried, pearl millet, untreated pearl millet, untreated

sorghum, which is not known to cause goiter, methanol-extracted pearl millet and methanol-extracted pearl millet plus ferulic acid. Weight gains and feed efficiencies were calculated to assess any anti-nutrient properties of the methanol extract or the fereulic acid. Goiter-inducing effects of the diets were evaluated on the basis of thyroid hormone concentrations and histological changes in the thyroid glands.

The methanol extract was analyzed by paper chromatography, UV spectroscopy and high performance liquid chromatography (HPLC). The amount of ferulic acid in each diet was quantitated using HPLC. An HPLC procedure was developed to separate and quantitate glucosylorientin and glucosylvitexin plus vitexin.

Results of the feeding study indicated that the methanol extract was goitrogenic. All animals fed sorghum plus pearl millet methanol extract developed goiter-like thyroid histological abnormalities. Animals fed extracted pearl millet exhibited thyroid hormone patterns more similar to animals fed sorghum in comparison to those fed untreated millet. The methanol extract also appeared to contain an anti-nutrient. The ferulic acid did not appear to act as agoitrogen, or as an anti-nutrient. Wetting the millet apparently increased the anti-nutrient and goitrogenic activity of

the methanol extract. It is possible that flavones act as the goitrogen or as a precursor of a goitrogenic compound.